University of Alberta

Influence of the Endoplasmic Reticulum Environment on Calreticulin Structure and Function

by

Elaine Francisca Corbett

A thesis submitted to the Faculty of Graduate Studies in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Biochemistry

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Elaine Corbett

Tubbermurry,
Pallaskenry,
Co. Limerick
Ireland

August 31, 2001

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Influence of the Endoplasmic Reticulum Environment on Calreticulin Structure and Function" submitted by Elaine Francisca Corbett in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dr. Marek Michalak (supervisor)

Dr. Chris Bleackley

Dr. David Brindley

Dr. Tom Hobman

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To my mother and father, Eileen and Michael Corbett, for the continuous love, support and encouragement that they have given me throughout my life.

Go raibh míle maith agaibh agus tá grá agam duit!

ABSTRACT

Calreticulin is one of the major, Ca^{2+}-binding chaperones in the lumen of the endoplasmic reticulum (ER). The protein functions both as a lectin-like chaperone and as a modulator of Ca^{2+} homeostasis. I investigated interactions between calreticulin and other ER resident chaperones (PDI and ERp57), with a special emphasis on the role of the ER luminal ionic environment. ER luminal proteins PDI and ERp57 were labeled with a conformational specific fluorescent dye Cascade Blue. Changes in the fluorescence intensity of the labeled proteins correspond to conformational changes in the proteins and therefore were used to report conformational-dependent protein:protein interactions. Using this approach I found that calreticulin interacts with PDI in a Ca^{2+}-dependent manner. In the absence of Ca^{2+} the proteins form complexes, that dissociate upon the re-addition of Ca^{2+}. Interaction between calreticulin and ERp57 requires a Zn^{2+}-dependent conformational change in ERp57 followed by a Ca^{2+}-dependent stabilization of the complex.

Interaction of calreticulin with PDI or ERp57 occurs *via* the N-domain of calreticulin, whereas the Ca^{2+} sensitivity/modulation of these interactions is confined to the high capacity Ca^{2+} binding, C-domain of the protein. I have also established that calreticulin is an ATP-binding protein. This was accomplished using ATP affinity chromotography and limited proteolytic digestion. Calreticulin binds to the affinity column and is specifically eluted with high concentrations of ATP. In the presence of ATP, the protein is also highly resistant to proteolysis implying ATP-induced conformational changes in calreticulin.

Limited proteolytic digestion with trypsin was used to monitor how alterations in the ER lumenal environment effects the conformation of calreticulin. Digestion of calreticulin alone results in rapid proteolysis. Binding of Ca^{2+} or Zn^{2+} to calreticulin results in the generation of different protease-resistant fragments indicating that binding of Ca^{2+}, Zn^{2+} and ATP (which are known to be present in the ER lumen) or interaction with other chaperones induces conformational changes in calreticulin. These results are consistent with calreticulin being flexible in solution and with its role as a molecular chaperone.

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LIST OF ABBREVIATIONS AND NOMENCLATURE USED

α	alpha
Aβ	β-amyloid protein
AD	alzheimers disease
ADP	adenosine diphosphate
AMP	adenosine monophosphate
APP	amyloid precursor protein
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
β	beta
BiP	immunoglobulin heavy chain binding protein
β2-m	β2-microglobulin
Ca^{2+}	calcium
CALNUC	calcium-binding protein nucleobindin
CB	cascade blue
CD	circular dichroism
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator protein
CGN	*cis*-Golgi network
CHO	chinese hamster ovary
CTLs	cytotoxic T-lymphocytes
DAG	diacylglycerol
DBD	DNA binding domain
DEPC	diethyl pyrocarbonate
DTPA	diethylenetriaminepentaacetic acid
DTT	dithiothreitol
EGTA	ethylenediaminetetraacetate
ER	endoplasmic reticulum
ERAD	ER membraneassociated degradation
ERp57	endoplasmic reticulum protein 57

ERp60	endoplasmic reticulum protein 60
ERSE	endoplasmic reticulum stress response element
ES	embryonic stem cell
FITC	fluorescein isothiocyanate
FPLC	fast performance liquid chromotography
free $[Ca^{2+}]_{ER}$	the concentration of free Ca^{2+} in the ER lumen
free $[Ca^{2+}]_C$	the concentration of free Ca^{2+} in the cytosol
FRET	fluorescence resonance energy transfer
γ	gamma
GDP	guanosine triphosphate
GFP	green fluorescent protein
Glc	glucose
$GlcNAc_2$	N-acetyl-glucosamine
G protein	guanosine triphosphate-binding protein
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GRP94	glucose regulated protein 94
GTP	guanosine triphosphate
HACBP	high affinity calcium binding protein
hCG-β	human chorionic gonadotropin β
$InsP_3$	inositol 1,4,5-triphosphate
Ire1p	inositol response element protein 1
KDa	kilodalton
Man	mannose
MEF	mouse embryonic fibroblast
Mg^{2+}	magnesium
MHC	myosin heavy chain
mM	milimolar
MOPS	4-morpholinepropanesulfonic acid
mRNA	messenger ribonucleic acid
MW	molecular weight

ND	not detectable
NES	nuclear export signal
NF-AT	nuclear factor of activated T-cell
NF-κB	nuclear factor κB
NK	natural killer cells
NLS	nuclear localization signal
nM	nanomolar
PC	phosphatidylcholine
PDI	protein disulfide isomerase
PE	phosphatidylethanolamine
PERK	protein endoplasmic reticulum kinase
PI	phosphatidylinositol
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PLC-β	phospholipase C beta
PM	plasma membrane
PS	phosphatidylserine
RER	rough endoplasmic reticulum
RNA	ribonucleic acid
RNase	ribonuclease
RyR	ryanodine receptor
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SERCA	sarcoplasmic and endoplasmic reticulum calcium ATPase
SR	sarcoplasmic reticulum
SRP	signal recognition particle
UGGT	UDP-glucose: glycoprotein glucosyltransferase
UP	unfolded proteins
UPR	unfolded protein response
VWD	von Willibrands disease
WT	wild-type
Zn^{2+}	zinc

Protein Abbreviations

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
X		any amino acid

Chapter One

Introduction

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Endoplasmic Reticulum

Introduction

Unlike prokaryotic cells, which generally consist of a single intracellular compartment surrounded by a plasma membrane (PM), eukaryotic cells are elaborately subdivided into functionally distinct, membrane-bound compartments. Each compartment, or organelle, contains its own characteristic set of enzymes and other specialized molecules, and complex distribution pathways allow the transport of specific products from one compartment to another. One such organelle is the endoplasmic reticulum (ER). The ER is one of the largest membrane organelles in eukaryotic cells, and typically constitutes more than half of the total membrane of the average animal cell (Carroll, 1989; Kaufman, 1999). This membrane plays a role in a variety of cellular processes. The ER membrane is the site of synthesis of both membrane proteins and secreted proteins, in addition to being the main dynamic Ca^{2+} storage compartment of the cell. It also plays a central role in lipid biosynthesis. Most of the membrane components in eukaryotic cells are synthesized in the ER. In fact, it is the site of production of lipids not only for the ER itself, but also the Golgi apparatus, lysosomes, endosomes, secretory vesicles and the PM (Alberts, 1994). Production of nearly all of the lipids required for the elaboration of new cell membranes, including both phospholipids and cholesterol occurs in the ER. Synthesis of phospholipids and cholesterol takes place on the cytoplasmic side of the ER, where all the necessary metabolites are found. The major phospholipids produced in the ER are phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS), with phosphatidylinositol (PI) being produced as a minor lipid. Phospholipid translocators in the ER membrane are responsible for the transfer of some

of the newly formed phospholipid molecules from the cytosolic side of the ER membrane to the luminal side. An ER translocator preferentially recognizes and transfers choline-containing phospholipids and is therefore responsible for the asymmetric distribution of lipids in the bilayer. This organelle is also involved in the production of ceramide, which is then exported to the Golgi apparatus (Alberts, 1994). Although lipid production is certainly one of the major functions of the ER, the focus of my introduction will be on the role of the ER in calcium storage and homeostasis and protein synthesis as these are relevant to my field of research.

The ER is a major protein folding compartment in the cell – it is involved in the synthesis, post-translational modification and folding of secreted, PM and organelle proteins. The lumen of the ER is a dynamic environment perfectly designed for the task of protein folding. It contains the largest concentration of chaperones, the majority of which are Ca^{2+}-binding proteins, and provides an optimal environment for protein folding, modification and assembly (Meldolesi and Pozzan, 1998a). It is also considered one of the most important and metabolically relevant sources of cellular Ca^{2+} (Pozzan *et al.*, 1994; Clapham, 1995). The concentration of free Ca^{2+} (free $[Ca^{2+}]_{ER}$) is within the range of a few hundred micromolar, which is at least three orders of magnitude higher than in the cytosol (Montero *et al.*, 1995, Miyawaki *et al.*, 1997; Montero *et al.*, 1997; Meldolesi and Pozzan, 1998a; Robert *et al.*, 1998). The concentration of free Ca^{2+} in the cytosol (free $[Ca^{2+}]_C$) of any resting cell is extremely low (~ 100 nM), however, upon agonist stimulation, Ca^{2+} is released from the ER and the free $[Ca^{2+}]_C$ can rise up to ~ 1,000 nM (Bootman and Berridge, 1995; Ashby and Tepikin, 2001). This is significantly lower than the 2 mM $[Ca^{2+}]$ found extracellularly (Clapham, 1995). It is widely accepted

that Ca^{2+}, when released into the cytosol, is an extremely important signaling molecule. For this signaling mechanism to work, the resting free $[Ca^{2+}]_C$ must be kept low. This is achieved in several ways; namely by the active pumping of Ca^{2+} from the cytosol to the ER or to the cell exterior and by the use of various molecules that buffer Ca^{2+} in the cell by binding free Ca^{2+} tightly.

The ER lumen, the internal space, comprises over 10% of the cell volume (Alberts, 1994; Pahl, 1999). It is therefore not surprising that the ER plays a central role in a panoply of cellular functions, but it is generally agreed that two of its major functions in the cell and those that are to be discussed in greater detail are: - Ca^{2+} homeostasis and protein biosynthesis.

Role of the Endoplasmic Reticulum in Ca^{2+} homeostasis

The ER is considered to be the main dynamic Ca^{2+}-storage compartment in the cell (Meldolesi and Pozzan, 1998a). Alterations in intracellular Ca^{2+} homeostasis have profound effects on many cellular functions. In many cells types, increases in free $[Ca^{2+}]_C$ is due to release from intracellular stores such as the ER. The ER is a major signal transducing organelle within the cell that continuously responds to environmental cues to release Ca^{2+}. The release of Ca^{2+} from intracellular stores is initiated by the binding of many hormones and growth factors to specific receptors on the PM. Binding of these receptors by an extracellular ligand alters the conformation of the cytoplasmic domain of the receptor, causing it in turn to bind to a G protein (trimeric GTP-binding proteins). A G protein is composed of three different polypeptide chains, called α, β, and γ. The G_α subunit binds to and hydrolyzes GTP. The G_β and G_γ subunits form a tight complex ($\beta\gamma$), which anchors the G protein to the cytoplasmic side of the PM. A G protein that is GTP-

bound is said to be active, while a G protein that is GDP-bound is said to be inactive. G proteins serve to functionally couple specific receptors to their target enzymes in the PM. Once stimulated, by binding of an activated receptor, the G_{α} subunit binds to and hydrolyzes GTP, allowing dissociation from $G_{\beta\gamma}$ subunits. The G protein then induces the activation of a PM enzyme, leading to activation of further downstream messengers. Upon activation, the G protein, Gq, activates phospholipase C-β (PLC-β). Both G_{α} and $G_{\beta\gamma}$ bind to and lead to the activation of PLC-β (Clapham and Neer, 1993; Clapham, 1995; Neer, 1995). PLC-β then catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) leading to the production of the intracellular messengers' inositol 1,4,5-triphosphate ($InsP_3$) and diacylglycerol (DAG). $InsP_3$ is highly mobile in the cytoplasm of the cell and it diffuses rapidly to the cell interior where it binds to specific receptors that are present on the ER membrane. Several types of messenger-activated channels mediate the release of Ca^{2+} from intracellular stores.

Ca^{2+} is released from the ER by $InsP_3$ receptor and/or Ryandine receptor (RyR) Ca^{2+} release channels and it is taken up by the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (McPherson and Campbell, 1993; Coronado *et al.*, 1994; Furuichi and Mikoshiba, 1995; Sutko and Airey, 1996; MacLennan *et al.*, 1997). The binding of $InsP_3$ to the $InsP_3$ receptor induces changes in the conformation of the receptor, so that an integral channel is opened thus allowing Ca^{2+}, stored at high concentrations in the ER, to be released into the cytoplasm. The $InsP_3$ and RyR receptors are structurally similar and both are subject to positive feedback regulation, whereby modest concentrations of released Ca^{2+} can bind to the receptors to further increase Ca^{2+} release. Therefore Ca^{2+} is released from the ER in a sudden, "all or nothing" manner. Some Ca^{2+} also enters from

the extracellular environment by crossing the PM (Bootman and Berridge, 1995; Clapham, 1995). The majority of Ca^{2+} released into the cytosol is subsequently transported back into the lumen of the ER *via* SERCA. The PM Ca^{2+}-ATPase and the Na^{+}-Ca^{2+} exchanger also remove Ca^{2+} from the cytosol (Carafoli, 1987; Pietrobon *et al.*, 1990; Clapham, 1995).

The release and re-uptake of Ca^{2+} results in a continuous fluctuation of the free $[Ca^{2+}]_{ER}$ (Miyawaki *et al.*, 1997; Meldolesi and Pozzan, 1998a). When Ca^{2+} is taken up from the cytoplasm into the ER, the Ca^{2+} stores are considered to be full; when Ca^{2+} is released from the ER into the cytoplasm, the Ca^{2+} stores are considered empty.

Due to the importance of the ER as being the main source of intracellular Ca^{2+}, there has been considerable attention and debate as to the exact free $[Ca^{2+}]_{ER}$. The total concentration of Ca^{2+} in the ER lumen is estimated to be 1-3 mM (Meldolesi and Pozzan, 1998a). A significant portion of this Ca^{2+} is free, but the free $[Ca^{2+}]_{ER}$ is difficult to measure and thus remains a point of considerable controversy. Depending on the method used values as low as 1 μM and as high as 3 mM has been reported (Meldolesi and Pozzan, 1998a). Elegant studies using fusion-protein constructs of green-fluorescent protein (GFP) and blue-fluorescent protein known as 'cameleons' have given a more definitive answer to this question (Miyawaki *et al.*, 1997). Cameleons consist of tandem fusions of a blue- or cyan-emitting mutant of GFP, calmodulin, the calmodulin-binding peptide M13, and an enhanced green- or yellow-emitting GFP. Binding of Ca^{2+} to calmodulin, induces calmodulin to wrap around the M13 domain, bringing the two flanking GFPs closer together thus leading to an increase in the fluorescence resonance energy transfer (FRET). These cameleons display biphasic Ca^{2+}-binding with two

dissociation constants, thus giving it the ability to report a wide range of $[Ca^{2+}]$. Mutations of calmodulin can further tune the Ca^{2+} sensitivity so that free $[Ca^{2+}]$ in the range of 10^{-8} to 10^{-2} can be measured. Cameleons can be targeted to different intracellular organelles; with targeting to the ER lumen facilitated by the addition of the ER signal sequence. The inclusion of a KDEL ER retrieval signal ensures that the cameleon is retained in the ER. By this method it was demonstrated that when Ca^{2+} stores are full, the free $[Ca^{2+}]_{ER}$ ranges from 60-400 μM. Upon agonist stimulation and hence depletion of the stores, the free $[Ca^{2+}]_{ER}$ ranged from 1-50 μM (Miyawaki *et al.*, 1997). The relatively large range of values reported in these studies may reflect differences in the cell types used and also heterogeneity in the environment of the ER lumen. However, these studies do demonstrate that, during agonist-dependent depletion of the ER stores, the free $[Ca^{2+}]_{ER}$ fluctuates from as low as 1 μM and as high as 400 μM (Miyawaki *et al.*, 1997).

There is an enormous range of known responses to increases in free $[Ca^{2+}]_C$, some with a duration of a few seconds, others where the response is far more prolonged (Bootman and Berridge, 1995; Clapham, 1995; Meldolesi and Pozzan, 1998a). Alterations in the intracellular Ca^{2+} concentration regulate a variety of diverse cellular functions including secretion, contraction-relaxation, cell motility, cytoplasmic and mitochondrial metabolism, protein synthesis, modification and folding, gene expression, cell cycle progression and apoptosis (Little *et al.*, 1994; Pozzan *et al.*, 1994; Clapham, 1995; Ghosh and Greenberg, 1995). More recently, it has become apparent that Ca^{2+} stored in intracellular organelles can also be an important regulator of intracellular functions. Changes in the free $[Ca^{2+}]_{ER}$ affect many functions of the ER, including the synthesis and secretion of proteins, the interactions of chaperones with each other and

with their substrates and the activation of Ca^{2+} influx *via* PM channels (Lodish *et al.*, 1992; Lodish and Kong, 1990; Sambrook, 1990; Baksh *et al.*, 1995a; Vassilakos *et al.*, 1998). Consequently, the ability of the ER membrane to control Ca^{2+} homeostasis has profound effects on many cellular functions. The ER and its lumen contain a characteristic set of resident proteins that are involved in every aspect of the ER function and Ca^{2+} homeostasis is no exception (Nash *et al.*, 1994).

ER luminal calcium buffers

Within the ER, most Ca^{2+} is buffered by the abundant luminal Ca^{2+}-binding proteins that are present there (Table 1-1). Many ER resident proteins are Ca^{2+}-binding proteins, having a weak affinity for Ca^{2+}, but with a high binding capacity. The first Ca^{2+}-binding protein identified was calsequestrin in striated muscle sarcoplasmic reticulum (SR) (MacLennan and Wong, 1971; Campbell *et al.*, 1983b). Calsequestrin is the most abundant Ca^{2+}-binding protein in the SR and is therefore thought to represent the major Ca^{2+} buffer of the SR. Calsequestrin is a high capacity Ca^{2+}-binding protein, whose very acidic C-terminal domain binds Ca^{2+} with low affinity (K_D = 1 mM) and high capacity (B_{max} = ~50 moles of Ca^{2+}/mole of protein) (MacLennan and Wong, 1971; Campbell *et al.*, 1983a; Mitchell *et al.*, 1988). Several years later, a second major Ca^{2+}-binding protein was identified. Calreticulin was first isolated as a high affinity Ca^{2+}-binding protein of the skeletal muscle SR by Ostwald and MacLennan in 1974 (Ostwald and MacLennan, 1974). The protein was observed to bind Ca^{2+} with high affinity and was therefore named the high affinity Ca^{2+}-binding protein (HACBP). Calreticulin does not contain the

Table 1-1: Ca^{2+}-binding proteins in the lumen of the ER

Protein name	Alias	Ca^{2+}-binding *moles Ca^{2+}/mole protein*	Ca^{2+}-binding sites
Calreticulin	See Table 1-2	~25 1*	clusters of acidic amino acid residues P-rich helix-loop-helix motif
Calnexin	p88	1*	P-rich helix-loop-helix motif
Calsequestrin	ERcalcistorin/calstorin	~50	clusters of acidic amino acid residues
BiP	GRP78	1-2	acidic amino acid residues
GRP94	Endoplasmin ERp99/HSP108	10	clusters of acidic amino acid residues
PDI	ERp59/T3BP/GSBP	19	clusters of amino acid residues
ERp72	ERp78	Still unclear	
ERp57	ERp61/p5/GRP58, Phosphatidylinositol specific PLC	ND	
Calmegin	Meg 1	1*	P-rich helix-loop-helix motif

characteristic EF-hand domains associated with high affinity Ca^{2+}-binding proteins. Calreticulin contains two calcium-binding sites (Ostwald and MacLennan, 1974; Baksh and Michalak, 1991). The protein binds Ca^{2+} with affinity and low capacity (B_{max} = 1 mole of Ca^{2+}/mole protein; K_D = 1 μM) and as well with low affinity and high capacity (B_{max} = >25 moles Ca^{2+}/mole protein; K_D = 2 mM) (Ostwald and MacLennan, 1974; Baksh and Michalak, 1991). Since then, many ER resident proteins have been identified that display high Ca^{2+}-binding capacity (tens of Ca^{2+} ions per molecule of protein) with low affinity (K_D in 1 mM range). Like many other abundant ER luminal proteins, Glucose regulated protein 94 (GRP94) is a low-affinity, high-capacity Ca^{2+}-binding protein (Koch *et al.*, 1986; Macer and Koch, 1988; Van *et al.*, 1989). GRP94, like calreticulin, displays both high and low affinity Ca^{2+}-binding sites. GRP94 is believed to have 15 Ca^{2+}-binding sites, four with high affinity (K_D ~ 2 μM) and eleven with low affinity binding (K_D ~ 600 μM) but high capacity (B_{max} = 10 moles of Ca^{2+}/mole of protein) (Macer and Koch, 1988; Van *et al.*, 1989). GRP94's Ca^{2+}-binding sites are believed to consist of highly negatively charged regions distributed throughout the length of the protein. Protein disulfide isomerase (PDI) is another Ca^{2+}-binding protein of the ER membranes. Again EF hand motifs are absent from PDI (Macer and Koch, 1988), but many of the acidic residues in PDI interact with Ca^{2+}. PDI displays a weak affinity for Ca^{2+} (K_D ~ 2.77 – 5.2 mM), but has a high capacity for binding (B_{max} = 19 moles of Ca^{2+}/mole of protein) (Lebeche *et al.*, 1994). Calnexin has also been shown to bind Ca^{2+} (Wada *et al.*, 1991; Cala *et al.*, 1993). Calnexin is a type-1 transmembrane phosphoprotein, and is the only membrane-spanning chaperone identified so far. Its luminal domain shares striking similarity to calreticulin (Bergeron *et al.*, 1994; Helenius

et al., 1997). Calnexin, like calreticulin, is a lectin-like chaperone, both of which recognize mono-glucosylated nascent proteins and will be discussed in greater detail later on in this chapter. BiP, another major ER luminal chaperone has also been shown to play an important role in the storage of Ca^{2+} within the ER lumen (Macer and Koch, 1988; Lievremont *et al.*, 1997). BiP has an abundance of acidic amino acids in its sequence that allow it to bind 1-2 moles of Ca^{2+}/mole of protein (Ting *et al.*, 1987; Lievremont *et al.*, 1997).

It is now known that changes in the free $[Ca^{2+}]_{ER}$ can affect many functions of the ER. Ca^{2+} also alters the activity of many ER resident proteins. Ca^{2+} can induce conformational changes in GRP94 (Van *et al.*, 1989), and has been shown to regulate interactions of GRP94 with other proteins (Koyasu *et al.*, 1989). Ca^{2+}-binding to PDI results in conformational changes in the protein that can be detected spectroscopically (Lebeche *et al.*, 1994). BiP is another example of an ER resident chaperone whose activity is altered by Ca^{2+}. BiP's ATPase activity is altered by Ca^{2+} and the association of BiP with proteins *in vitro* is stabilized by Ca^{2+} (Kassenbrock and Kelly, 1989; Gaut and Hendershot, 1993a; Gaut and Hendershot, 1993b). Changes in $[Ca^{2+}]_{ER}$ also affects calreticulin function. Interaction of calreticulin with PDI is Ca^{2+}-dependent (Baksh *et al.*, 1995a). Calreticulin undergoes dynamic, Ca^{2+}-dependent interactions with newly synthesized proteins and with ER proteins involved in Ca^{2+} transport (Camacho and Lechleiter, 1995; John *et al.*, 1998). These studies suggest that changes in the free $[Ca^{2+}]_{ER}$ may also play a signaling role in the lumen of the ER and that Ca^{2+}-binding to these chaperones could be involved in 'sensing' these changes.

Koch originally proposed to name the proteins in the lumen of the ER by the collective name 'Reticuloplasmins', to reflect their internal localization (Koch, 1987). It soon became evident that many of these reticuloplasmins were responsible for buffering Ca^{2+} in the ER lumen. One significant feature of the SR is that its luminal proteins, including calsequestrin, do not exist as freely diffusible species, but instead are part of an immobile network (Koch, 1987). Studies on ER membranes demonstrated that the reticuloplasmins similarily do not appear to be freely diffusible species but instead organize into some higher order structure. For this reason and the fact that many of the reticuloplasmins do not appear to have uniform distribution within the ER lumen, Koch proposed that the reticuloplasmins also may not be freely diffusible species and may be part of some supra-molecular complex. Since the reticuloplasmins are Ca^{2+}-binding proteins, it was proposed that Ca^{2+} might play an important role in the formation of such intra-luminal structures. The reticuloplasmins are believed to weakly interact together through what is described as a 'calcium-matrix' (Koch, 1987; Sambrook, 1990). This 'calcium-matrix' may undergo reversible sol-gel transformations dependent on cellular Ca^{2+} oscillations. This would allow the maintenance of a coherent network of chaperones as an immobile intraluminal 'calcium-matrix' and would leave an interspace mobile fluid phase. In this fluid phase, protein folding intermediates would be concentrated, facilitating their association with chaperones and folding enzymes and therefore enhancing protein folding (Hammond and Helenius, 1995; Tatu and Helenius, 1997). Modulation of the free $[Ca^{2+}]_{ER}$ would provide a mechanism to retain in the organelle both resident proteins and newly synthesized secretory proteins that are not folded and assembled into a transport competent state. Once assembled, the secretory protein could

then dissociate from their respective chaperone, presumably freeing them to leave the matrix and enter transport vesicles to their final cellular destination. In contrast, the Ca^{2+}-binding ER resident proteins may dissociate from one another but would be largely retained in the lumen of the ER. Any resident proteins that did leave the ER could be retrieved by the action of their KDEL ER retrieval signal. Therefore in addition to a signaling role, fluctuating Ca^{2+} levels in the ER lumen may also play a role in interaction of chaperones with each other and with substrate proteins.

Role of ER in the synthesis and folding of proteins

For a protein to perform it's correct function, it must be folded into a distinct three-dimensional conformation, and remain folded for a biologically appropriate time. The characteristic fold of each protein is determined by its amino acid sequence, a concept known as Anfinsen's dogma (Anfinsen, 1961b). Nearly all polypeptides in eukaryotic cells are translated in the cytosol, but many fold into functional, mature proteins only after transport to other compartments. Thousands of proteins reside in the PM of human cells, within membranous organelles or in extra cellular spaces. All of these proteins share a common origin in the ER, the compartment where they fold. Approximately one-third of all cellular proteins are translocated into the lumen of the ER where post-translational modification, folding and oligomerization occur (Kaufman, 1999). The ER membrane is the site of production and folding of all integral membrane proteins for most of the cell organelles, including the ER itself, Golgi apparatus, lysosomes, endosomes, secretory vesicles and the PM. Also, proteins that are destined for secretion from the cell are initially delivered to the ER lumen.

It has been realized for many years that many polypeptides can fold spontaneously and can reform native structures easily by themselves *in vitro* (Anfinsen, 1961a). Work by Anfinsen *et al*, demonstrated that denatured RNase can fold spontaneously in a test-tube without the addition of any other co-factors or helper enzymes and give rise to a biologically active enzyme (Anfinsen, 1961a). RNase is a relatively simple, monomeric protein and renaturation *in vitro* takes place in ~ 20 minutes (Goldberger, 1963). However, the time it takes for a protein to fold *in vitro* is too slow to be functional in cells. Renaturation of multi-domain proteins therefore could take several hours *in vitro*. The total number of conformations available for a protein sequence to fold is enormous - if a protein were to sample all possible conformations then folding would be extremely slow. This is best illustrated by 'Levinthal's paradox' (Levinthal, 1968), which states that if a given amino acid can assume approximately 10 different conformations, the total number of possible conformations in a polypeptide chain of 100 residues would be 10^{100}. Obviously this speed of folding is too slow for cellular requirements – so what actually happens *in vivo*? It soon became apparent that protein folding *in vivo* is facilitated by proteins contained in the ER of eukaryotic cells, and that some proteins (complex, multi-domain or oligomeric proteins) could only fold and assemble efficiently in the presence of these additional proteins; molecular chaperones. So why do we need molecular chaperones? The experimental conditions required to successfully fold many proteins *in vitro* is usually very constrictive – low protein concentration, long incubation periods and temperatures that are not physiologically relevant. Folding in the cell on the other hand takes place in a crowded cellular environment - folding is occurring simultaneously with thousands of other proteins and

therefore the risk of aggregation for the folding polypeptide is obviously great. In addition, folding occurs in the presence of very high concentrations of cellular metabolites, membranes and other cellular components. However there are exceptions as examination of the folding pathway of hCG-β (human chorionic gonadotropin) subunit intermediates demonstrated that the *in vitro* folding pathway was indistinguishable from the intracellular folding pathway (Huth *et al.*, 1993). However, the rate and efficiency with which proteins achieve their final native state *in vivo* is higher than *in vitro,* a difference credited to the molecular chaperones. Molecular chaperones prevent the aggregation and misfolding of newly synthesized polypeptides, prevent non-productive interactions with cellular components and direct the assembly of other cellular components.

Protein synthesis in the cell is complex (Gething and Sambrook, 1992). The polypeptide gradually becomes exposed as it emerges from the ER membrane-bound ribosome and is translocated into the cisternal space of the ER. Cleavage of the signal sequence peptide and addition of N-linked oligosaccharides occurs co-translationally. Processing of the N-linked oligosaccharides takes place and formation of disulfide bonds occurs, facilitated by the unique oxidizing environment provided by the ER. In the case of multimeric proteins, oligomerization and subunit assembly occurs. Quality control and selective degradation systems ensure that only conformationally mature proteins are transported out of the ER. Proteins that are destined for the cell surface or secretion are transported to the Golgi apparatus where further processing occurs. The proteins are then either transported to the cell surface or packaged into secretory vesicles for secretion.

The lumen of the ER is a dynamic environment perfectly designed for the task of protein folding. There are several aspects of the ER luminal environment that make it unique - the ER is an oxidizing environment, it has high Ca^{2+} concentrations and proteins translocated into the ER lumen are subject to glycosylation (Helenius, 1992).

Co- and post-translational modification

During translation, proteins are translocated into the ER lumen through a pore in the ER membrane. The ER, therefore, is the first compartment of the secretory pathway through which newly synthesized proteins must pass. Most proteins that fold in the ER are targeted there by an N-terminal signal sequence (Blobel and Dobberstein, 1975). Signal peptides were originally discovered in the early 1970's by Blobel *et al* (Blobel and Dobberstein, 1975). In these experiments mRNA encoding a secreted protein was translated by ribosomes *in vitro*. They found that if microsomes were omitted from this cell-free system, a protein was synthesized that was slightly larger than the normal secreted protein. If microsomes were present in this experiment, then a protein of the correct size was produced. The difference in size between the two protein species was due to the presence of an amino-terminal leader peptide. This led Blobel to propose the 'Signal peptide hypothesis'- which postulates that the amino-terminal leader peptide directs the protein to the ER membrane and is then cleaved off by a signal peptidase in the ER membrane before polypeptide chain synthesis is completed (Blobel and Dobberstein, 1975).

Signal sequences play a key role in targeting and membrane insertion of secretory and membrane proteins. Signal sequences are typically 15-30 amino acids long and

display no obvious sequence homology, but they do share the common feature of having a high proportion of hydrophobic amino acids (Carroll, 1989). Upon initiation of translation, and as soon as a typical 20 amino acids signal sequence protrudes from the large subunit of the ribosome, it is bound by a signal recognition particle (SRP). The SRP cycles between the ER membrane and the cytosol and is responsible for guiding the signal sequence-containing peptide to the ER membrane. Upon binding of the SRP to the ER signal peptide, protein translation pauses, presumably allowing the ribosome enough time to reach the ER membrane and preventing the release of the peptide into the cytosol. Once formed the SRP-ribosome complex binds to the SRP receptor, a rough endoplasmic reticulum (RER) integral membrane protein. The SRP is released and the nascent polypeptide is translocated into the ER lumen through a pore in the ER membrane. The signal sequence is cleaved by a signal peptidase and is rapidly degraded by proteases in the cytosol (Martoglio and Dobberstein, 1998).

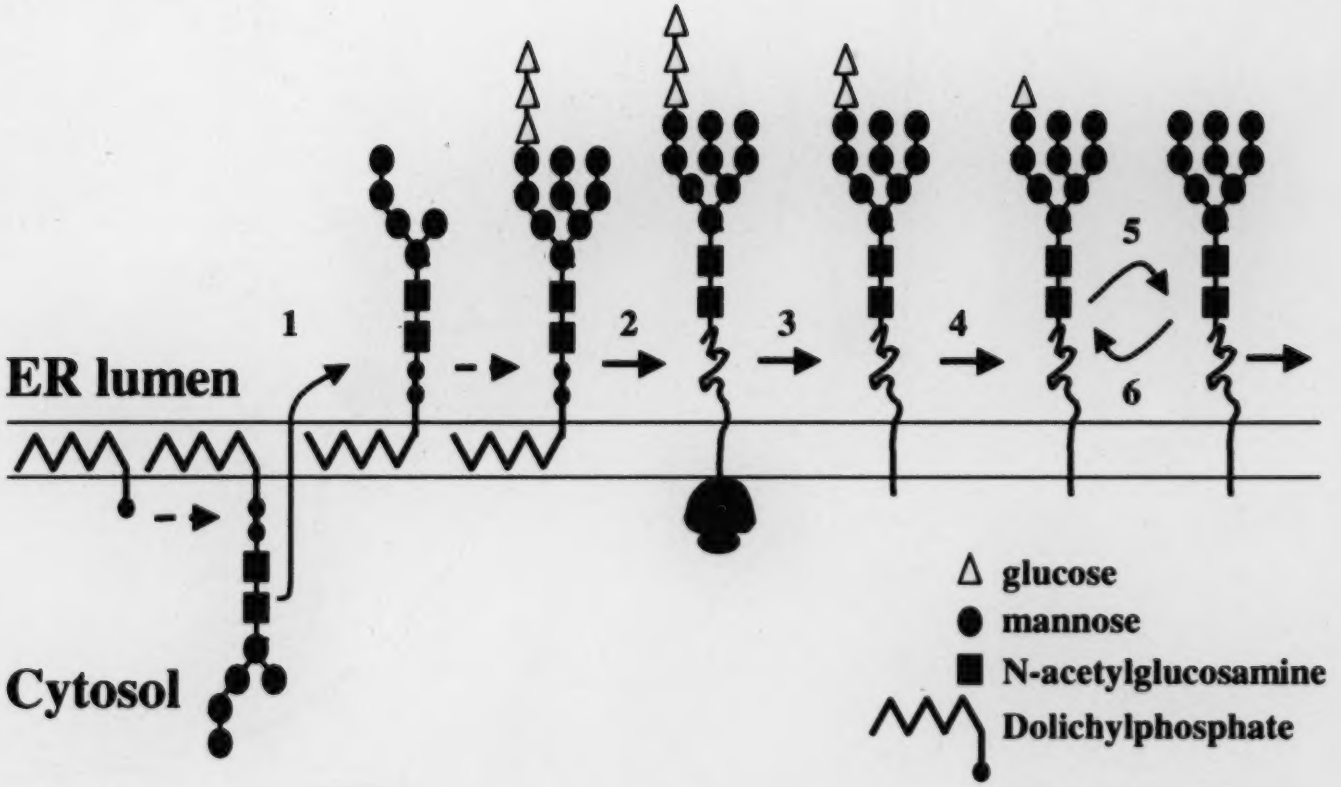
The protein is released into the ER lumen, where it undergoes a variety of co- and post-translational modifications. One such modification is glycosylation. Almost as soon as the polypeptide chain enters the ER lumen, it is glycosylated on target amino acids. The addition of N-linked (Asn-linked) oligosaccharides to a nascent polypeptide serves a number of functions in the cell. One such function is that it promotes interaction of the nascent polypeptide with specific ER luminal chaperones, and thereby enhances folding. The majority of soluble and membrane-bound proteins synthesized in the ER, including those destined for other cellular locations e.g. Golgi apparatus, lysosomes, PM and extracellular space, are glycoproteins. Synthesis of N-linked oligosaccharides begins on the cytosolic side of the ER membrane by the addition of sugars to a lipid molecule called

dolichylphosphate (Refer to Fig. 1-1). Initially two N-acetylglucosamines and five mannose residues are added and the oligosaccharide is flipped to the luminal side of the ER membrane (Fig. 1-1 (**1**)). Once in the ER lumen an additional four mannose and three glucose sugars are added to the oligosaccharide complex. The preformed oligosaccharide (consisting of two N-acetyl-glucosamine, nine mannose (Man) and three glucose (Glc) moieties ($GlcNAc_2$)) is transferred *en bloc* to the NH_2 group of an asparagine (Asn) amino acid in the protein, and is said to N-linked or Asn-linked (Fig. 1-1 (**2**)). Oligosaccharide addition generally occurs when the acceptor Asn residue is ~ 12-14 amino acids from the membrane (Nilsson and von Heijne, 1993). The transfer of the sugar residue is catalyzed by a membrane-bound enzyme an oligosaccharyl transferase, which has its active site exposed on the luminal side of the membrane.

After transfer of the pre-assembled oligosaccharide ($Glc_3Man_9GlcNAc_2$) to the nascent polypeptide at selected Asn residues in the sequence Asn-X-Ser/Thr, the newly synthesized proteins then undergo a series of alterations in three-dimensional structure as well as post-translational modification (Parodi, 2000a). The mature protein as it exits the ER will no longer contain these three glucose residues. The outmost glucose moiety on the core oligosaccharide is quickly removed in the ER by glucosidase I, followed by the sequential removal of the two remaining glucose by glucosidase II. Refer to Figure 1-1 (**3**), (**4**), and (**5**). During this period of glycan trimming, glycoproteins obtain their correct tertiary and quaternary structure and if the glycoprotein is correctly folded it escapes the folding cycle. The folding is assisted by numerous molecular chaperones and mediated by isomerases (Helenius *et al.*, 1997). Correctly folded proteins exit the ER, while incorrectly folded proteins have been found to remain in the ER lumen. If the protein is

Figure 1-1: Biosynthesis of N-linked core oligosaccharides

Synthesis begins on the cytosolic side of the ER membrane with the addition of sugars, one by one to dolichylphosphate. As soon as two N-acetylglucosamines and five mannoses have been added, the oligosaccharide is flipped to the ER lumenal side of the membrane (1). An additional four mannose and three glucose sugars are added to the oligosaccharide, after which the core oligosaccharide is transferred to the asparagine residue of the nascent protein (2). The glucoses are trimmed away by glucosidase I (3) and glucosidase II (4 & 5). If the glycoprotein is correctly folded it leaves the ER for the Golgi apparatus. If the glycoprotein is not fully folded it is recognized as such by UGGT and is re-glucosylated (6). Adapted from (Helenius and Aebi, 2001)

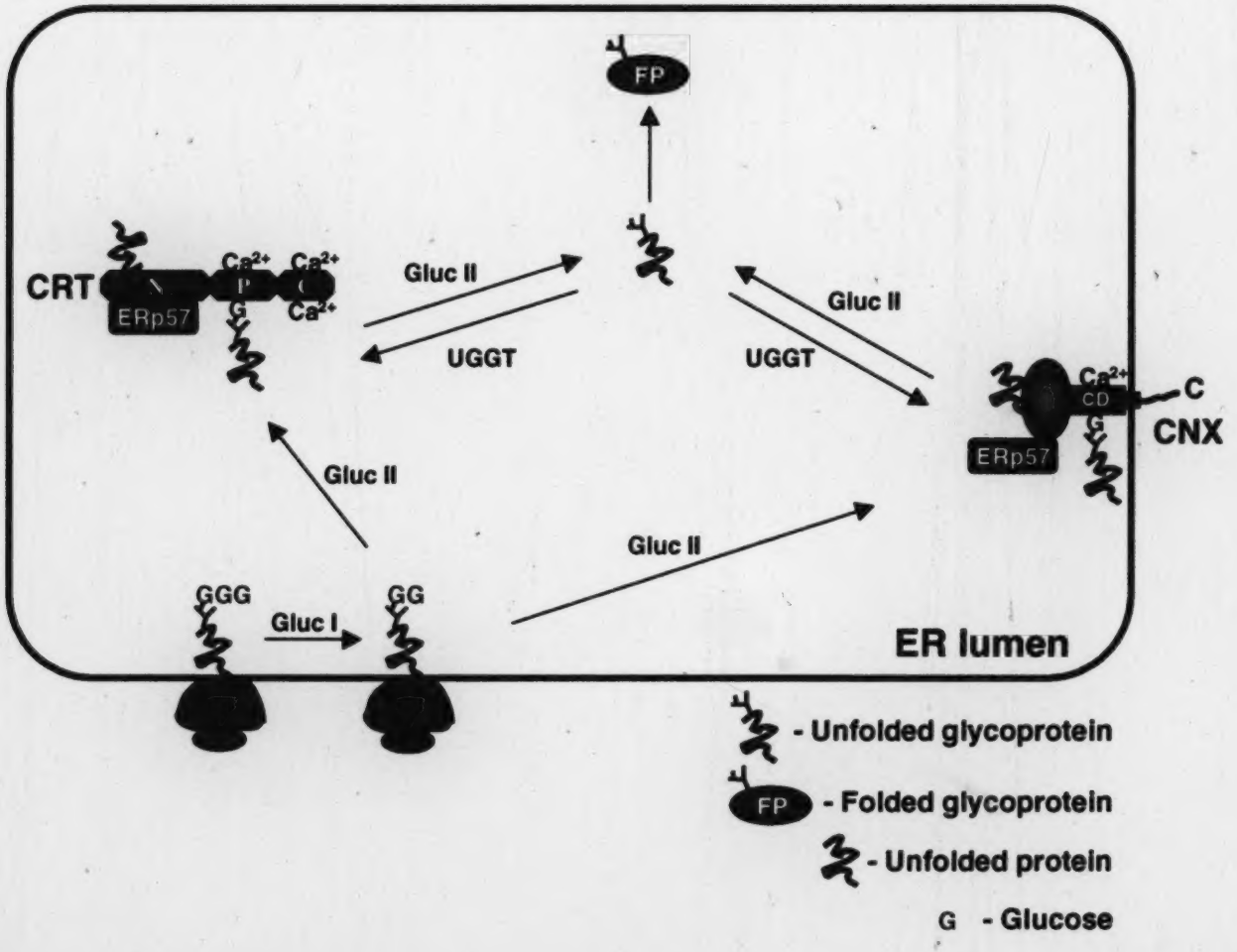

ER lumen
Cytosol
1
2
3
4
5
6
glucose
mannose
N-acetylglucosamine
Dolichylphosphate

not correctly folded, a single glucose residue is added to non-glucosylated misfolded glycoproteins by the enzyme UDP-glucose: glycoprotein glucosyltransferase (UGGT) (Fig. 1-1 (**6**))(Sousa and Parodi, 1995). UGGT is a sensor for the folding state of glycoproteins and can discriminate between folded and in-correctly folded substrates (Sousa and Parodi, 1995). More recent studies have demonstrated that UGGT poorly recognizes fully unfolded polypeptides, but distinguishes between different non-native conformations and shows a distinct preference for structured folding intermediates (Trombetta and Helenius, 2000). The re-addition of this terminal glucose residue by UGGT tags the incorrectly folded glycoprotein, allowing interaction of calreticulin and calnexin with the glycoprotein, thus promoting proper folding (Hebert *et al.*, 1996; Van Leeuwen and Kearse, 1996b; Van Leeuwen and Kearse, 1997; Wada *et al.*, 1997). Refer to Figure 1-2.

Calreticulin and calnexin are two homologous ER chaperones that specifically bind monoglucosylated core oligosaccharides (Spiro *et al.*, 1996; Vassilakos *et al.*, 1998). In doing so, they promote proper folding and assembly of glycoproteins, while also restricting exit of newly synthesized proteins from the ER to those that have attained their final native conformation (Fig. 1-2). In addition to their lectin-like function, both calreticulin and calnexin behave as true molecular chaperones, interacting with non-glycosylated folding intermediates (Ihara *et al.*, 1999; Saito *et al.*, 1999). Glucosidase II will indiscriminately remove the most terminal glucose moiety. If the glycoprotein is correctly folded it will leave the cycle and leave the ER for the Golgi apparatus where further processing will occur. If the glycoprotein is unfolded, it is re-glucosylated and is re-bound by calreticulin or calnexin. Therefore, unfolded glycoproteins undergo cycles of

Figure 1-2: The calreticulin and calnexin chaperone cycle

As soon as two of the glucoses in the N-linked core glycans ($Glc_1Man_9GlcNAc_2$) have been trimmed away by the actions of glucosidase I (Gluc I) and glucosidase II (Gluc II), interaction with calreticulin (CRT) and/or calnexin (CNX) occurs. Unfolded glycoproteins undergo cycles of binding to, and release from, calreticulin and calnexin. The glucose moiety is removed by Gluc II, and if the glycoprotein is correctly folded it leaves the cycle. If the glycoprotein is not correctly folded, the terminal glucose is once again attached by UGGT. If the glycoproteins contain cysteines the formation of disulfide bonds is catalyzed by ERp57, a protein that displays increased activity in the presence of either calreticulin or calnexin. The cycle is repeated until either the protein is folded or degraded. Once the glycoprotein is correctly folded it is no longer recognized by UGGT, therefore is not reglucosylated, and will not longer bind to calreticulin or calnxin, and exits the ER for the Golgi apparatus.


FP
CRT
ERp57
P
Ca2+
Ca2+
Ca2+
G
Gluc II
UGGT
Gluc II
UGGT
Ca2+
CD
C
CNX
ERp57
Gluc II
Gluc II
GGG
Gluc I
GG
ER lumen
- Unfolded glycoprotein
FP
- Folded glycoprotein
- Unfolded protein
G - Glucose

interaction with calnexin and calreticulin, until folding is completed and the peptide is ready to leave the ER. This process is known as the calreticulin-calnexin cycle. If the glycoprotein does not reach its native conformation in the ER, it is transported to the cytosol and eliminated by ER-associated degradation (ERAD) (Brodsky and McCracken, 1999; Plemper and Wolf, 1999).

ER luminal conditions

Several conditions make the ER a unique folding compartment. This arises from the different nature of proteins that fold in the ER, the machinery that translocates them across the ER lumen, and from the physiological functions of the ER. These aspects of the ER environment necessitate the presence of enzymes and chaperones to facilitate folding in the ER, and indeed the most abundant luminal proteins (BiP, GRP94, PDI and calreticulin) all function in protein folding (Fink, 1999).

Unlike proteins in the cytosol, proteins folded in the ER are rich in cysteines (Cys) and usually possess multiple disulfide bonds (Gilbert, 1990). The oxidizing conditions of the ER lumen allow the formation of disulfide bonds, a protein modification that is generally disfavored in the reducing environment of the cytosol. The major redox buffer in the ER lumen is the Cys-containing tripeptide glutathione (Hwang *et al.*, 1992). The ratio of reduced to oxidized glutathione ranges from 1:1 to 3:1, whereas the overall cellular ratio ranges from 30:1 to 100:1. How exactly this oxidative environment is maintained is not completely clear, however, the preferential transport of glutathione from the cytosol to the ER lumen is likely to be a contributing factor (Hwang *et al.*, 1992). Disulfide bonds are very important in determining protein folding pathways as

they form covalent folding intermediates and therefore can restrict the number of available conformations for a folding intermediate (Creighton, 1997). Disulfide bonds can form at different times during protein folding, sometimes forming as soon as the available Cys residues are in the ER lumen, and sometimes later in the folding pathway (Marquardt and Helenius, 1992; Segal *et al.*, 1992). In contrast, some proteins can assume the same native structure, even if oxidation occurs during folding (co-) or after (post-) translation. For example, treatment of cells with reducing agents such as dithiothreitol (DTT) prevented disulfide bond formation of the asialoglycoprotein receptor and reduced the oxidized proteins present in the ER (Lodish *et al.*, 1992). When the reducing agents were washed out, the reduced proteins rapidly oxidized and folded correctly. These results indicate that oxidation of disulfide bonds appears to occur in the same manner whether it occurs co- or post-translationally. Therefore, disulfide bonds in most cases may serve to stabilize a local fold rather than initiating the folding process itself. To support this, mutagenesis experiments show that lysozyme can be reasonably well folded even without some of its disulfide bonds (Taniyama *et al.*, 1992). Human lysozyme is a secretory protein containing four disulfide bonds. A mutant lysozyme, in which the two Cys residues at amino acid positions 75 and 95 are replaced with alanine (Ala), displays an overall tertiary structure and enzymatic activity that is essentially identical to the wild-type (WT) protein. These results indicate that this disulfide bond is unnecessary for the folding and maintenance of the structure of the protein. However, removal of this disulfide bond strongly accelerates the unfolding of lysozyme (by four orders of magnitude) indicating that its removal remarkably destabilizes the protein. There are several luminal enzymes that are involved in disulfide bond formation. The

most abundant of the ER disulfide isomerases are PDI and two structurally related proteins, ERp72 and ERp57. There is currently no evidence for the existence of disulfide isomerases in any other organelle.

ATP is required to support correct folding and disulfide bond formation in many proteins, and is an important component of the ER luminal environment (Flynn *et al.*, 1989; Dorner *et al.*, 1990; Braakman *et al.*, 1992; Clairmont *et al.*, 1992; Dorner and Kaufman, 1994; Wei *et al.*, 1995; Wei and Hendershot, 1995; Guthapfel *et al.*, 1996; Berninsone and Hirschberg, 1998). ATP is required in the ER lumen for protein phosphorylation (Hendershot *et al.*, 1988; Leustek *et al.*, 1991; Rindress *et al.*, 1993; Cala, 1999; Cala and Jones, 1994; Quemeneur *et al.*, 1994; Csermely *et al.*, 1995; Chen *et al.*, 1996) and protein degradation (Hirschberg *et al.*, 1998). Many chaperones of the ER bind and utilize ATP. For example, both BiP and PDI bind and hydrolyze ATP (Munro and Pelham, 1986; Flynn *et al.*, 1989; Wei *et al.*, 1995; Wei and Hendershot, 1995; Guthapfel *et al.*, 1996). ATP binding and hydrolysis by BiP is required for release of associated proteins from BiP and therefore their secretion (Flynn *et al.*, 1989; Dorner and Kaufman, 1994; Wei *et al.*, 1995; Wei and Hendershot, 1995). In addition, several other ER luminal chaperones including GRP94 have been demonstrated to bind ATP (Clairmont *et al.*, 1992). However, neither ATP binding nor hydrolysis is necessary for its peptide binding activity (Wearsch and Nicchitta, 1997). ATP binding to calnexin induces conformational changes in the protein, as assessed by an increased sensitivity to proteolysis and also induces protein oligomerization (Ou *et al.*, 1995). Although calnexin binds ATP it does not display any ATPase activity (Ou *et al.*, 1995). An ATP transporter has been identified in the ER lumen (Clairmont *et al.*, 1992). However, attempts to

measure the levels of ATP present in the ER lumen have thus far been unsuccessful (Dorner and Kaufman, 1994).

Many soluble, membrane-bound, as well as proteins destined for other cellular locations, are glycoproteins. The addition of carbohydrates to nascent polypeptide chains serves multiple roles in protein folding. Carbohydrates are hydrophilic in nature and therefore increase the solubility of the glycoprotein. They generally mark the surface of folding modules and are not buried within them and they make the process of translocation across the ER membrane less reversible. Carbohydrates can also affect protein folding in other ways, in that they allow and specify interactions of newly synthesized glycoproteins with the lectin-like chaperones calreticulin and calnexin.

The ER is the major Ca^{2+} store in the cell and therefore undergoes quite rapid changes in Ca^{2+} levels, depending on the metabolic needs of the cells (Meldolesi and Pozzan, 1998a). Thus, protein folding in the ER lumen must be able to adapt to Ca^{2+} fluctuations. Individual proteins can respond differently to changes in $[Ca^{2+}]$: depletion of Ca^{2+} has no effect on the folding or secretion of newly synthesized albumin, but totally blocks asialoglycoprotein receptor maturation (Lodish *et al.*, 1992). Thyroglobulin folding is also retarded when the ER Ca^{2+} stores are perturbed (Kuznetsov *et al.*, 1997). Many Ca^{2+}-binding proteins in the ER lumen also serve as molecular chaperones involved in the folding and quality control of ER proteins. Perturbation of the ER luminal Ca^{2+} levels affects the activity of many of these proteins (Suzuki *et al.*, 1991; Zhang *et al.*, 1997).

ER chaperones and folding enzymes

Proteins synthesized in the ER that are destined to enter the early secretory pathway to the Golgi apparatus most certainly have a difficult trip ahead of them. Both the ER membrane and lumen are often densely packed with proteins, some of which are incompletely folded or unassembled units of multi-subunit complexes. The danger of aggregation is obviously higher for newly synthesized polypeptides, which expose hydrophobic segments that in the mature protein would be buried in the native structure. Avoiding aggregation is the first challenge – here come in the molecular chaperones and folding enzymes. Numerous resident proteins reside within the ER through a mechanism that requires their continuous vesicle-mediated retrieval from post-ER compartments within the early secretory pathway. Some of these ER resident proteins are chaperones and catalysts of protein folding that form a matrix on which newly synthesized proteins attain their final conformation. There are several classes of proteins that forming this environment specialized in folding, assembly and sorting (Refer to Table 1-1).

Firstly, there are the proteins that catalyze the specific isomerization steps that may otherwise limit the rate of folding of some proteins. An example of such a protein would be PDI, which catalyzes protein-folding reactions and increases the rate of protein folding, without actually changing the pathway through which proteins attain their final folded conformation. PDI catalyzes the oxidation of disulfides and isomerizes incorrect (or correct) disulfides on nascent polypeptides undergoing folding in the oxidizing environment of the ER (Noiva and Lennarz, 1992; Freedman *et al.*, 1994). PDI belongs to a family of protein-thiol oxidoreductase enzymes that includes ERp57 and ERp72 and which have sequence and structural similarity to thioredoxin (Edman *et al.*, 1985). ERp57

is a homologue of PDI with thiol-dependent reductase (Hirano *et al.*, 1995) and cysteine-dependent reductase activities (Urade *et al.*, 1997). ERp72 possesses both redox and disulfide-isomerase activity (reviewed in (Ferrari and Söling, 1999), and has been implicated in ER proteolytic activity (Urade *et al.*, 1993).

Secondly, there are protein chaperones that do not actively catalyze protein folding, but instead maintain proteins in a folding-competent state. They prevent aggregation of protein-folding intermediates and stabilize conformations of polypeptides that are energetically unfavorable in order to minimize irreversible "dead end" protein folding (Dill and Chan, 1997). One of the most abundant and well-characterized ER chaperones is BiP (reviewed in (Gething, 1999). BiP was originally identified as the immunoglobulin heavy chain binding protein (Haas and Wabl, 1983) and as the glucose-regulated protein, GRP78 (Pouyssegur *et al.*, 1977). BiP binds transiently to newly synthesized proteins in the ER and for longer periods to misfolded, underglycosylated or unassembled proteins whose transport from the ER is blocked. BiP does not bind to native polypeptides (Gething, 1999). BiP has two major domains, an N-terminal domain that contains the ATPase catalytic site and a C-terminal domain that binds substrates (McKay, 1993). These two domains communicate to regulate the affinity and duration of polypeptide binding so that unfolded polypeptides chaperoned by BiP undergo cycles of binding and release that is coupled to the binding and hydrolysis of ATP (Flynn *et al.*, 1989). BiP binds to early folding intermediates from a vast array of proteins, due to its ability to recognize short hydrophobic peptide segments (Flynn *et al.*, 1989). BiP is also involved in a variety of other ER functions including translocation of newly synthesized polypeptides across the ER membrane (Brodsky and McCracken, 1999; Plemper and

Wolf, 1999). BiP interacts in a conformation- and ATP-dependent manner with Sec63, a major component of the ER translocation pore, where it mediates import of nascent proteins from the cytoplasm (Corsi and Schekman, 1997; McClellan *et al.*, 1998). Once BiP binds the ER luminal J domain of Sec63, this interaction induces rapid hydrolysis of ATP giving rise to an ADP-bound BiP. Hydrolysis of ATP converts the open peptide-binding pocket of BiP into a closed pocket, resulting in ADP-BiP binding to the translocating peptide. This allows rapid trapping of peptides, which are then slowly released upon nucleotide exchange (Lyman and Schekman, 1997; Matlack *et al.*, 1999). Multiple BiP molecules are transferred to each substrate molecule increasing the efficiency of translocation. BiP therefore acts as a molecular ratchet, binding the translocating substrate on the luminal side of the membrane, and preventing its from moving backwards, but without hindering its forward movement. BiP dissociates from the substrate peptide following nucleotide exchange. Binding of ATP re-opens the peptide-binding pocket, allowing release of substrate (Lyman and Schekman, 1997; Matlack *et al.*, 1999). BiP also plays a role in the transport of misfolded proteins across the ER membrane for degradation (ERAD) by the proteosomes in the cytosol. In common with ER import of protein, export during ERAD also utilizes the translocation complex and depends on the participation of BiP (reviewed in (Brodsky and McCracken, 1999; Lord *et al.*, 2000). However, studies have indicated that the role of BiP during protein import and export is different and that the two processes are mechanistically distinct (Brodsky *et al.*, 1999).

Another abundant but one of the least understood chaperones of the ER lumen is GRP94. In contrast to BiP, GRP94 has been demonstrated to associate with relatively few

proteins (Argon and Simen, 1999). GRP94 has been shown to interact transiently with a number of peptides and unfolded proteins, including unassembled immunoglobulin light and heavy chains (Melnick *et al.*, 1994). GRP94 appears to preferentially associate with more advanced folding intermediates, rather than earlier folding intermediates. GRP94 also displays peptide-binding activity and associates with a number of antigenic peptides. GRP94 is involved in the transport of peptides for antigenic presentation and therefore plays an important role in both immunity and tumor biology (Nieland *et al.*, 1996; Argon and Simen, 1999). Its expression is up-regulated by the presence of unfolded proteins in the ER (Kozutsumi *et al.*, 1988).

The molecular chaperone composition of the ER differs from other folding compartments in the fact that it also contains a unique type of chaperones - the lectin-like chaperones that bind to mono-glucosylated proteins. Two such ER chaperones are known to exist, calreticulin, a lumenal protein, and calnexin, a membrane-spanning chaperone, and these will be discussed in greater detail in the calreticulin section of this chapter.

All of these ER resident proteins are selectively retained in the ER lumen either due to the fact that they contain a specific KDEL ER retrieval/retention sequence. Many ER molecular chaperones have been shown to co-associate or act contemporaneously on nascent proteins to facilitate their folding.

Quality control (QC)

Proteins enter the secretory pathway through the ER *en route* to their final cellular destination. Protein folding in the ER lumen is not completly efficient and some polypeptides will not achieve correct folding, particularly if the cell is under some form

of cellular stress or if mutations have been induced into the polypeptide. It is now well established that if folding in the ER is incomplete, transport of proteins out of the ER is inhibited (Ellgaard *et al.*, 1999; Parodi, 2000a; Parodi, 2000b). The ER has developed a 'quality control' system, which consists of a group of proteins that distinguish between native and incompletely folded proteins. These proteins continuously monitor for incompletely folded or aggregated intermediates, misfolded peptides or unassembled subunits and inhibit the traffic of these misfolded proteins to further cellular compartments or enhance their degradation. This is a very important process that avoids the export of functionally or structurally altered proteins from the ER. Chaperones and folding enzymes are clearly part of this system and the molecular chaperones BiP, PDI, GRP94, ERp57, ERp72, calreticulin and calnexin have all been implicated as part of the 'quality control process' in the ER lumen. Therefore, these proteins are not only responsible for assisting in the folding process itself, but also function as retention anchors for incompletely folded proteins. Association of newly synthesized protein with molecular chaperones and folding enzymes (QC factors) provides a primary QC mechanism ensuring that incompletely folded proteins do not reach further stages of the secretory pathway. Binding by any one of these resident proteins appears to be sufficient to prevent entry into the secretory pathway. This form of QC is based on the presence of a structural feature on the nascent protein that can be recognized by the molecular chaperones and folding enzymes present in the ER. These features are normally only transiently exposed on proteins, and therefore under normal cellular conditions only transient association with these QC factors occurs. This QC system displays a high degree of stringency as each of the QC factors recognize their substrates *via* a different

mechanism. Therefore if one chaperone fails to associate with an incompletely folded protein, another most likely will (Zhang *et al.*, 1997).

BiP has an ability to recognize a wide variety of unrelated nascent polypeptides, while actively discriminating between properly folded and unfolded structures. BiP is often found to associate preferentially with misfolded mutants, presumably because these molecules continue to expose hydrophobic binding sites that would normally be located in the interior of a fully folded protein (Blond-Elguindi *et al.*, 1993). BiP is capable of retarding the transport of substrates to further stages of the secretory pathway (Dorner *et al.*, 1992). GRP94 also displays peptide-binding activity and associates with folding intermediates, thereby working as part of the ER QC system (Nieland *et al.*, 1996)

Both calreticulin and calnexin are part of the QC system that prevents export of misfolded or incompletely folded proteins out of the ER. Both display an ability to interact with non-native polypeptide segments in unfolded proteins and thus are true molecular chaperones (Ihara *et al.*, 1999; Saito *et al.*, 1999; Jorgensen *et al.*, 2000). In a manner distinct from the classical chaperones, both calreticulin and calnexin also function as lectin-like chaperones, preventing the export of misfolded and incompletely folded glycoproteins from the ER (Ellgaard *et al.*, 1999). Calreticulin and calnexin serve as sensors of the glycosylation state of a protein *via* their specific binding to mono-glucosylated intermediates. They bind to almost all membrane-bound and soluble glycoproteins synthesized in the ER and together with ERp57, they mediate the retention and promote the folding of substrate proteins (Zapun *et al.*, 1998). Unlike other molecular chaperones, calreticulin and calnexin cooperate with several independently acting enzymes that regulate substrate binding. They co-operate with the enzyme that re-

attaches the terminal glucose, UGGT, a protein that distinguishes between folded and unfolded intermediates (Sousa *et al.*, 1992). They also cooperate with glucosidase II (a protein that is insensitive to the folding state of glycoproteins), the enzyme responsible for removing the terminal glucose. In this manner, the glycoprotein is recognized as immature, and is retained in the ER allowing further folding. Fully folded proteins can exit the calreticulin/calnexin cycle and proceed through the secretory pathway. Another mechanism of ER retention involves the formation of intermolecular disulfide-bonded complexes with PDI, ERp57 and ERp72 (Reddy and Corley, 1998). Yet another mechanism for the retention of newly synthesized proteins in the ER is by the formation of large aggregates. For example, MHC class II molecules form transient aggregates, to which BiP and calnexin associate, before acquiring their native structure (Marks *et al.*, 1995). Eventually, if the defect cannot be corrected, misfolded proteins are retro-translocated across the ER membrane back into the cytosol where, after deglucosylation and ubiquitination, they are digested by proteosomes (Kopito, 1997; deVirgilio *et al.*, 1998; Brodsky and McCracken, 1999; Lord *et al.*, 2000).

Quality control and human disease

The efficient synthesis and secretion of polypeptides is vital for intracellular communication and cellular function. It is therefore not surprising that mutations or environmental-induced defects in the synthesis, folding and secretion of specific polypeptides may lead to a variety of human diseases. The basic defect of many human diseases such as cystic fibrosis (CF), α1-antitrypsin and von Willibrands disease (VWD) involves the misfolding and ER retention of mutated but partially functional proteins.

This may be a major contributing factor to the development of disease pathology (Carlson, 1990; Thomas *et al.*, 1995; Aridor and Balch, 1999; Brodsky and McCracken, 1999; Bross *et al.*, 1999).

Wild-type CF trans-membrane conductance regulator protein (CFTR) is normally delivered to the PM, while ΔPhe508 CFTR, the most common mutation of CF, is retained in the ER. Both newly synthesized WT and ΔPhe508 CFTR specifically associate with calnexin. However, only WT CFTR is able to escape from this association and exit the ER (Chevet *et al.*, 1999). ΔPhe508 CFTR, like many other disease causing mutant proteins, is retained in the ER and eventually degraded by ERAD (Brodsky and McCracken, 1999; Bross *et al.*, 1999).

VWD is the most common bleeding disorder in humans. A novel VWD-causing mutation (Arg273Trp) results in defective multimerization and secretion of von Willibrands factor (VWF) (Allen *et al.*, 2000). Both WT and mutant VWF interact with calreticulin, calnexin and ERp57 during biogenesis in the ER. However, mutant VWF displays prolonged association with calnexin and ERp57 compared to WT, while there appears to be no apparent difference in association with calreticulin. The failure to detect a difference in binding of mutant protein to calreticulin in comparison to WT may be because the release of the mutant protein to the degradation pathway may be similar to the release of the WT protein from the ER (Allen, 2001).

There are also examples where the mutant protein is not degraded by ERAD, but remains in the cell. Alzheimers disease (AD) is characterized by the abnormal deposition of β-amyloid protein (Aβ), a peptide cleaved from the precursor protein amyloid precursor protein (APP). Examination of the localization and expression of calreticulin in

both normal and AD brains, revealed a lower level of expression of the protein in AD brains (Taguchi *et al.*, 2000). The expression level of BiP was the same in both cell types. It was suggested that calreticulin may be a multifunctional protein in the brain and that weak expression of calreticulin along with the positive staining of microglial processes in AD brains may be part of the pathological process. Calreticulin may be involved in the maturation of APP, and decreased expression of calreticulin may affect Aβ production in the ER. Another possibility is that decreased calreticulin levels may influence AD pathology by perturbing Ca^{2+} homeostasis. It has been proposed that early release of intracellular free Ca^{2+} exacerbates oxidative stress, and it has been suggested that oxidative stress may play an important role in AD. Over-expression of calreticulin blocks the increase in cellular Ca^{2+} and renders cells resistant to apoptosis caused by oxidative stress (Liu *et al.*, 1998). Oxidation of soluble Aβ causes its cross-linking and aggregation. Therefore oxidative stress due to decreased calreticulin content may play a role in AD pathogenesis (Taguchi *et al.*, 2000). Therefore an in-depth understanding of the ER molecular chaperones could provide insight into the role they play in genetic disease and therefore may aid in potential treatment.

Unfolded protein response (UPR)

When protein misfolding occurs in the ER, the cell initiates a number of processes. In both yeast and mammalian cells, the accumulation of misfolded polypeptides in the ER lumen results in the transcriptional up-regulation of many of the genes encoding ER resident chaperones and folding enzymes (for reviews see (Chapman *et al.*, 1998; Kaufman, 1999; Pahl, 1999). This unique intracellular signaling pathway is

known as the UPR and connects the ER with the nucleus. Cells respond to an increased level of unfolded proteins in the ER lumen by activating transcription of a set of genes involved in protein folding e.g. BiP, GRP94, calreticulin and PDI. The UPR can therefore adjust the protein folding capacity of the ER according to need, thus alleviating the increased demand on the existing cellular protein folding machinery. In addition to this, general protein translation is decreased, through phosphorylation of translation elongation initiation factor 2α (eIF-2α). Signaling occurs through two ER trans-membrane proteins, Inositol response element 1 protein (Ire1p) and protein endoplasmic reticulum kinase (PERK). The luminal domains of Ire1p and PERK recognize ER stress, while their cytosolic domains are involved in the specific cellular response that occurs. The luminal domains of these two proteins share significant homology, suggesting the existence of a similar ligand(s). Recent studies have shown that the luminal domain of the two proteins are functionally interchangeable, and found that in unstressed cells, both domains form stable complexes with the ER chaperone BiP (Bertolotti *et al.*, 2000). Perturbation of protein folding promoted the dissociation of BiP, allowing activation of both Ire1p and PERK. These finding are consistent with a model in which binding of BiP represses signaling through PERK or Ire1p. Protein misfolding relieves this repression by effecting the release of BiP from PERK and Ire1p luminal domains, thus allowing their activation.

Molecular chaperone and folding enzyme complexes

The exact mechanism by which molecular chaperones in the ER lumen facilitate folding is still largely unknown. Molecular chaperones are thought to interact with partially folded protein intermediates, while being continuously bound and released until

the polypeptide is completely folded. The folding and processing of polypeptides by the ER is thought to be a coordinated process. This belief is due to the identification of complexes consisting of folding intermediates in association with different molecular chaperones and due to their sequential action on common substrates. The unified action of molecular chaperones may function to hold a polypeptide in a specific conformation, to allow appropriate post-translational modifications or to drive the whole folding process so that the polypeptide attains a particular conformation. It is important during folding to prevent incorrect interactions of exposed protein domains, and molecular chaperones are thought to function in this role. One manner by which ER luminal proteins may associate to facilitate folding is through their Ca^{2+}-binding capabilities. As previously mentioned, the majority of reticuloplasmins are Ca^{2+}-binding proteins that are believed to interact weakly together through an immobile 'Ca^{2+} matrix' (Koch, 1987; Sambrook, 1990). The segregation of the ER into a membrane, along with an immobile network of chaperones would leave a mobile fluid space, facilitating interactions with folding enzymes and chaperones, thus facilitating protein folding. Oscillations in the free $[Ca^{2+}]_{ER}$ could provide a mechanism by which both resident proteins and newly synthesized secretory proteins could both interact and dissociate.

Numerous stable associations of multiple molecular chaperones with secretory proteins have been described. Chemical cross-linking experiments reveal that newly translocated polypeptides may interact with multiple ER luminal proteins (Kuznetsov *et al.*, 1997). BiP, GRP94, ERp72 and GRP170 can be isolated in complexes with newly synthesized thyroglobulin (Kuznetsov *et al.*, 1997). Similarly, cross-linking experiments also demonstrate that newly synthesized influenza haemagglutinin is part of a

heterogeneous complex that contains BiP, GRP94, calnexin, calreticulin and other proteins (Tatu and Helenius, 1997). Complexes of substrates with BiP or GRP94 or BiP and calnexin have been described (Melnick *et al.*, 1994). Similarly, ERp57 has been cross-linked to monoglucosylated glycoproteins that are substrates for calnexin and calreticulin (Elliott *et al.*, 1997; Oliver *et al.*, 1997). Further studies have identified functional complexes between ERp57, calreticulin and calnexin and demonstrated that the disulfide isomerase activity of ERp57 is much greater in the presence of calreticulin and calnexin suggesting a functional association between these proteins (Zapun *et al.*, 1998). However, upon initiation of these studies, direct interaction between calreticulin and ERp57 had not been demonstrated. These studies indicate that a variety of different molecular chaperones and folding enzymes act either simultaneously or sequentially during the conformational maturation of secretory proteins. The nature and sequence of involvement of the different molecular chaperones probably depends on the specific structural features displayed by the folding polypeptide during the different stages of folding and maturation.

Calreticulin

History of calreticulin

Calreticulin (HACBP) was first isolated as a high affinity Ca^{2+}-binding protein of the SR by Ostwald and MacLennan in 1974 (MacLennan *et al.*, 1971; Ostwald and MacLennan, 1974). This was followed many years later by its molecular cloning in 1989 by two groups; Smith and Koch and Fliegel *et al.* (Fliegel *et al.*, 1989a; Smith and Koch, 1989). It became apparent, after the amino acid sequence of HACBP was deduced from its cDNA, that several other calcium-binding proteins including CAB-63/calregulin and CBP58, discovered in or around the same time as calreticulin were in fact the same protein (Waisman *et al.*, 1985; Johnson *et al.*, 1992). Refer to Table 1-2. In addition, calreticulin was also known by several other names; CRP55 (Koch, 1987; Macer and Koch, 1988), CaBP3 (Peter *et al.*, 1992), ERp60 (Lewis *et al.*, 1985) and calsequestrin-like protein (Damiani *et al.*, 1988; Volpe *et al.*, 1988). Since the discovery of calreticulin there have been many proteins identified that are homologous to calreticulin; mobilferrin (Conrad *et al.*, 1990), Ro/SS-A autoantigen (McCauliffe *et al.*, 1990a; McCauliffe *et al.*, 1990b; Lux *et al.*, 1992), B50 (Gersten *et al.*, 1990), and C1q receptor (Malhotra, 1993; Malhotra *et al.*, 1993). All of the aliases of calreticulin are listed in Table 1-2. It is important to be aware of the aliases of calreticulin, as many of the biochemical studies conducted on calreticulin in the past have been under a different name. To avoid further confusion, the protein originally named HACBP was named 'calreticulin' to indicate that it is a calcium-binding protein (cal-) and is localized primarily to the ER (-reticulin). It became evident that calreticulin may play an important role in many cellular functions

Table 1-2: Calreticulin Identities

Name	Identified as	References
HACBP	Ca^{2+}-binding protein	(MacLennan, 1974; Ostwald and MacLennan, 1974)
CAB-63/Calregulin	Ca^{2+}-binding protein	(Waisman *et al.*, 1985)
CBP58	Ca^{2+}-binding protein	(Johnson *et al.*, 1992)
CRP55	Ca^{2+}-binding protein	(Koch, 1987; Macer and Koch, 1988)
CaBP3	Ca^{2+}-binding protein	(Peter *et al.*, 1992)
ERp60	ER cysteine protease	(Lewis *et al.*, 1985)
Calsequestrin-like protein	Ca^{2+}-binding protein	(Damiani *et al.*, 1988; Volpe *et al.*, 1988)
Mobilferrin	Fe^{3+}-binding protein	(Conrad *et al.*, 1990)
Ro/SS-A	SLE autoantigen	(Lux *et al.*, 1992; McCauliffe *et al.*, 1990a; McCauliffe *et al.*,1990b)
B50	Melanoma antigen	(Gersten *et al.*, 1990)
C1qR	C1q receptor	(Malhotra, 1993; Malhotra *et al.*, 1993)

and since that time the protein has been studied extensively by many laboratories (Michalak, 1996).

The calreticulin gene

To date cDNA and genes encoding calreticulin has been cloned from several vertebrates and invertebrates, and also in higher plants (Mazzarella *et al.*, 1992; Smith, 1992; Hawn *et al.*, 1993; Liu *et al.*, 1993; Nakamura *et al.*, 1993; Chen *et al.*, 1994; Huggins *et al.*, 1995; Crofts and Denecke, 1998; Navazio *et al.*, 1998). A gene encoding calreticulin has not been identified in prokaryotes or yeast. Evolutionary studies have revealed that the calreticulin gene is ~2 billion years old, existing prior to the divergence of mitochondrial eukaryotes ~1.5 billion years ago. Its membrane-spanning homologue, calnexin, is approximated to be ~1.6 billion years old (Llewellyn *et al.*, 2000). The protein is encoded by a single gene (McCauliffe *et al.*, 1992; Waser *et al.*, 1997) and only one species of 1.9 Kb mRNA encoding calreticulin has been identified (Fliegel *et al.*, 1989a). The calreticulin gene contains 9 exons, and spans approximately 3.6 KB or 4.6kb of human or mouse genomic DNA, respectively (McCauliffe *et al.*, 1992; Waser *et al.*, 1997). The nucleotide sequences of the mouse and the human gene show greater than 70% identity (McCauliffe *et al.*, 1992; Waser *et al.*, 1997) indicating a strong evolutionary conservation of the gene.

Regulation of expression of the calreticulin gene

The promoter of the mouse and human calreticulin genes contain several putative regulatory sites, including two AP-1 and eight AP-2 sites, GC-rich areas including three

Sp1 sites, a H4TF-1 site, and four CCAAT sequences (McCauliffe *et al.*, 1992; Waser *et al.*, 1997). A TATA box and an SIF PDGF binding site have also been identified (Waser *et al.*, 1997). AP-2 and H4TF-1 recognition sequences are typically found in genes that are active during cellular proliferation. Although calreticulin is expressed at low levels in the mature heart (Mesaeli *et al.*, 1999), calreticulin expression is up-regulated in the developing heart and has been shown to be essential for cardiac development (Mesaeli *et al.*, 1999). More recently Guo *et al* (Guo *et al.*, 2001) demonstrated that the cardiac specific transcription factor Nkx2.5 plays a central role in activating calreticulin expression in the developing heart. Binding of COUP-TF1 to the Nkx2.5 binding site suppresses transcription from the calreticulin promoter and may be involved in its post-natal suppression (Guo *et al.*, 2001).

The expression of calreticulin is affected by a number of different conditions and cellular events in different cell lines. Depletion of Ca^{2+} stores by the Ca^{2+} ionophore A23187, or thapsigargin, an inhibitor of SERCA, induces several-fold activation of the calreticulin promoter followed by concomitant increases in calreticulin mRNA and protein levels (Llewellyn *et al.*, 1995; Nguyen *et al.*, 1996; Waser *et al.*, 1997). An ER stress response element (ERSE) has also been identified in the calreticulin promoter (Yoshida *et al.*, 1998). p50ATF6, the active form of the transcription factor ATF6, can bind to this element, resulting in up-regulation of expression of calreticulin expression under conditions of ER stress i.e. UPR pathway (Yoshida *et al.*, 1998).

The calreticulin promoter is also activated by Zn^{2+} (Nguyen *et al.*, 1996) and heat shock (Conway *et al.*, 1995). Expression of calreticulin is also induced by viral infection (Zhu, 1996), cellular proliferation (Opas *et al.*, 1991), amino acid deprivation (Plakidou-

Dymock and McGivan, 1994; Heal and McGivan, 1998) and in stimulated cytotoxic T-cells (Burns *et al.*, 1992; Clementi *et al.*, 1994). This further indicates that the calreticulin gene is activated by a variety of chemical and biological stresses. Since calreticulin has been implicated in a wide variety of cellular processes, the stress-dependent activation of the calreticulin gene may affect numerous biological and pathophysiological conditions.

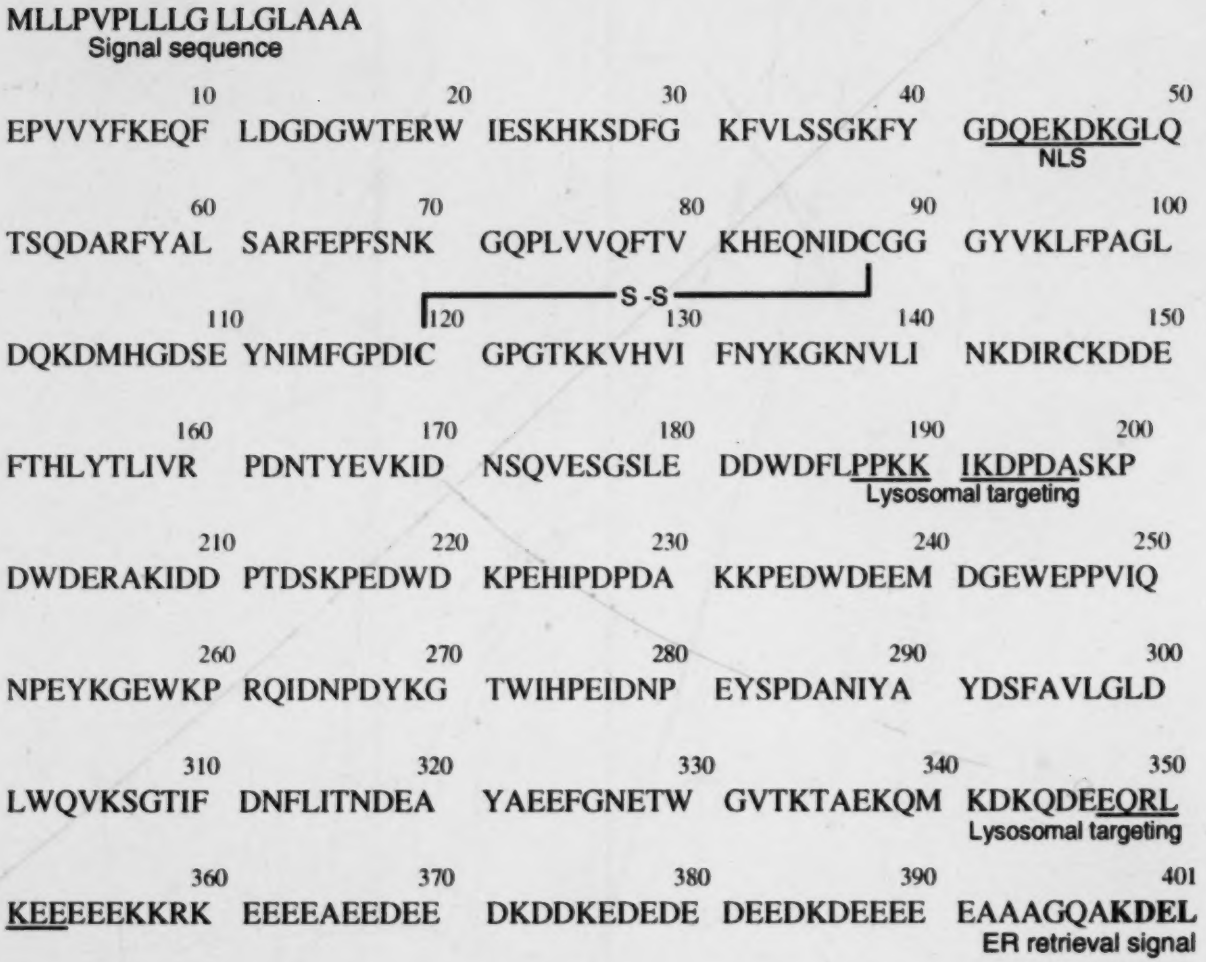
Amino acid sequence of calreticulin

The amino acid sequence of calreticulin was initially deduced from the nucleotide sequence of the cloned cDNA (Fliegel *et al.*, 1989a), and mature rabbit calreticulin exists as a protein of 401 amino acids. Please refer to Figure 1-3.

The MW of rabbit calreticulin as predicted from its amino acid sequence is 46,567 (Fliegel *et al.*, 1989a; Smith and Koch, 1989). However, upon analysis by SDS-PAGE (Laemmli system) calreticulin has a mobility corresponding to a 60 kDa protein (Waisman *et al.*, 1985; McCauliffe *et al.*, 1990a; Milner *et al.*, 1991). Mature, full-length calreticulin contains 109 acidic amino acids, most of which are located in the C-terminal region of the protein (Waisman *et al.*, 1985; McCauliffe *et al.*, 1990a). The discrepancy in MW is believed to be due to the high number of acidic amino acids in calreticulin. Discrepancies between predicted and observed motilities have been reported for several other proteins. Another example is calsequestrin, a Ca^{2+}-storage protein of the SR, which also has a highly charged C-terminal region (MacLennan, 1983). This discrepancy is again believed to be due to the high content of acidic amino acids that are present in the protein, as relatively low amounts of SDS would bind to these regions.

Figure 1-3. Rabbit skeletal muscle calreticulin amino acid sequence

The amino acid sequence of calreticulin as deduced from the nucleotide sequence of its rabbit cDNA clone ((Fliegel *et al.*, 1989)). Calreticulin contains 401 amino acids, beginning with an ER signal sequence and ending with a –KDEL ER retention/retrieval sequence. The putative nuclear localization signal (NLS) and lysosomal targeting sequences are underlined and labeled. Cysteine residues are shown in bold. Calreticulin contains a single disulfide bond between cysteine 88 and 120.

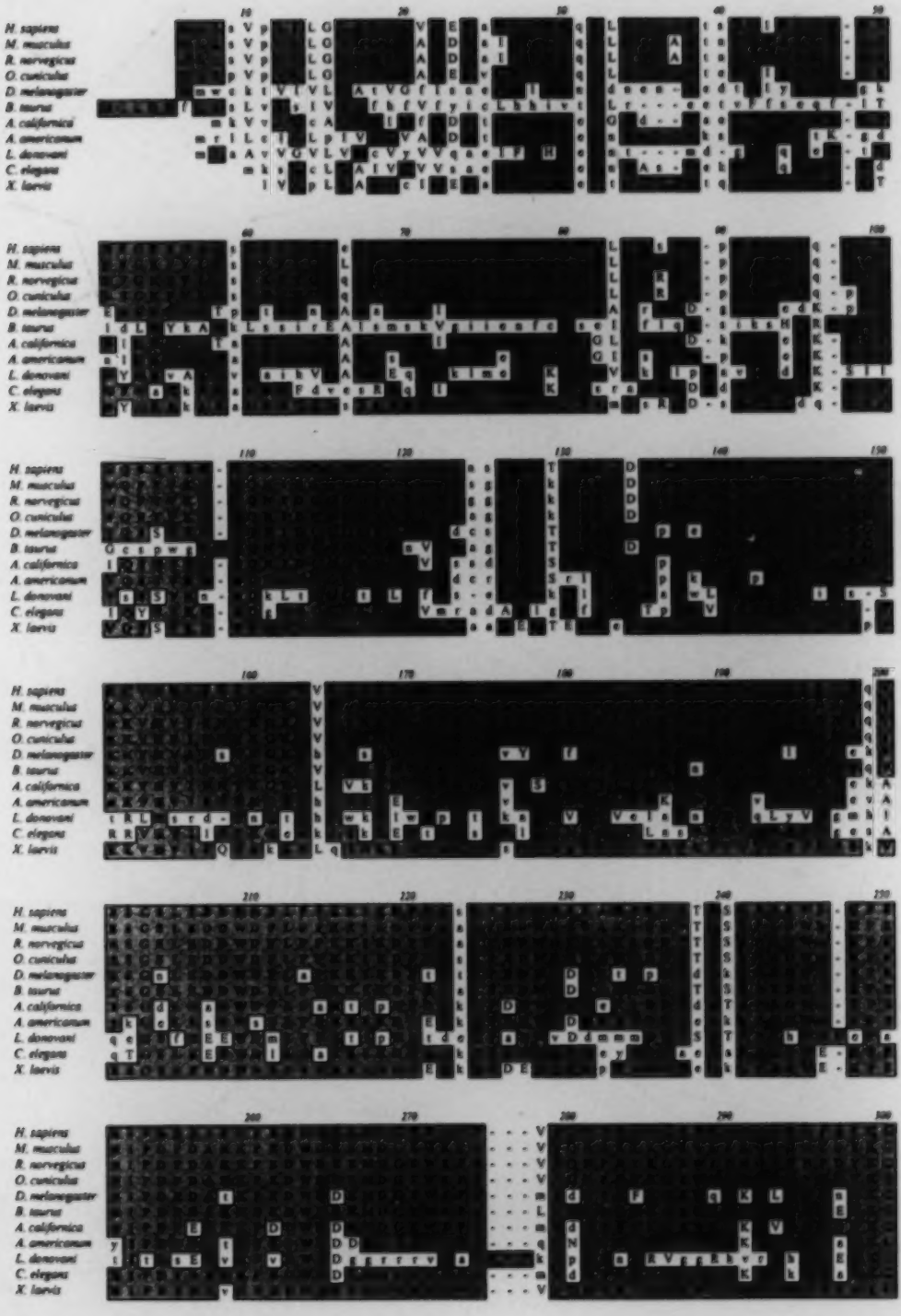

MLLPVPLLLG LLGLAAA
Signal sequence
10 20 30 40 50
EPVVYFKEQF LDGDGWTERW IESKHKSDFG KFVLSSGKFY GDQEKDKGLQ
NLS
60 70 80 90 100
TSQDARFYAL SARFEPFSNK GQPLVVQFTV KHEQNIDCGG GYVKLFPAGL
S-S
110 120 130 140 150
DQKDMHGDSE YNIMFGPDIC GPGTKKVHVI FNYKGKNVLI NKDIRCKDDE
160 170 180 190 200
FTHLYTLIVR PDNTYEVKID NSQVESGSLE DDWDFLPPKK IKDPDASKP
Lysosomal targeting
210 220 230 240 250
DWDERAKIDD PTDSKPEDWD KPEHIPDPDA KKPEDWDEEM DGEWEPPVIQ
260 270 280 290 300
NPEYKGEWKP RQIDNPDYKG TWIHPEIDNP EYSPDANIYA YDSFAVLGLD
310 320 330 340 350
LWQVKSGTIF DNFLITNDEA YAEEFGNETW GVTKTAEKQM KDKQDEEQRL
Lysosomal targeting
360 370 380 390 401
KEEEEEKKRK EEEEAEEDEE DKDDKEDEDE DEEDKDEEEE EAAAGQAKDEL
ER retrieval signal

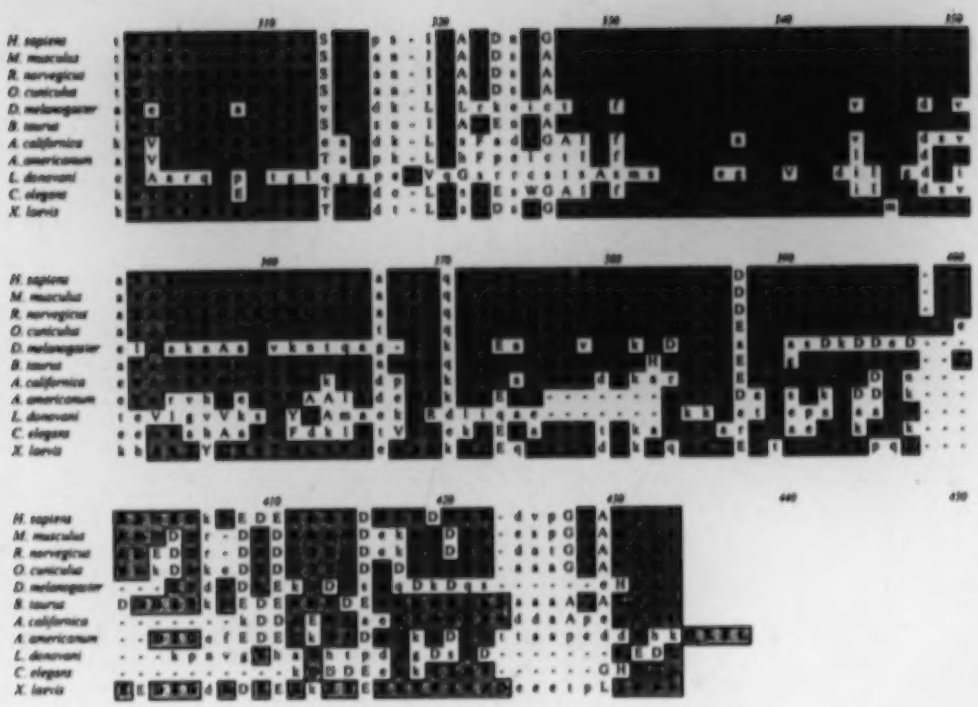
Analysis of the calreticulin amino acid sequence for consensus sequences or functional motifs reveals several important features. The protein starts with a cleavable N-terminal signal sequence that ensures targeting of the protein to the ER lumen (Fliegel *et al.*, 1989a; Rokeach *et al.*, 1991b; Denning *et al.*, 1997). The protein also possesses a putative nuclear localization signal (NLS) (McCauliffe *et al.*, 1990a; Opas *et al.*, 1991) as well as two lysosomal targeting signals (Dice, 1990), however it remains unknown if these targeting sequences are functional. The protein, like a number of other ER-resident proteins, including, PDI, BiP and GRP94, terminates with a KDEL ER retention signal (Pelham, 1989; Sonnichsen *et al.*, 1994).

Full-length amino acid sequences have been deduced for calreticulin from a variety of species and tissues and all display a high degree of amino acid sequence identity. Figure 1-4 shows an alignment of the calreticulin amino acid sequences from various species. Comparison of the amino acid sequences of human (McCauliffe *et al.*, 1990a; McCauliffe *et al.*, 1990b; Rokeach *et al.*, 1991a), mouse (Smith and Koch, 1989), rat (Murthy *et al.*, 1990), and rabbit (Fliegel *et al.*, 1989a) shows that there is over 90% amino acid sequence identity between the different species. Calreticulin from several other species also displays striking similarity. The *Drosophilia* calreticulin sequence displays over 80% identity to other calreticulins (McCauliffe *et al.*, 1990b). A presynaptic calcium-binding protein found in *Aplysia californica* is 72% identical to human calreticulin (Kennedy *et al.*, 1992). The amino acid sequence of Leishmania is 42% homologous to human calreticulin (Joshi *et al.*, 1996). The high degree of similarity in the amino acid sequences of calreticulin from the different species indicates that calreticulin is likely to serve important function/s within the cell.

Figure 1-4. Calreticulin amino acid alignment

Comparison of *H. sapiens, M. musculus, R. norvegicus, O. cuniculus, D. melanogaster, B. taurus, A. californica, A. americanum, L. donivani, C. elegans,* and *X. laevis* calreticulin amino acid sequences are shown. Identical residues are shown in bold uppercase with shading and outline, similarities are shown in uppercase, while mismatches are shown in lowercase.


H. sapiens
M. musculus
R. norvegicus
O. cuniculus
D. melanogaster
B. taurus
A. californica
A. americanum
L. donovani
C. elegans
X. laevis


H. sapiens
M. musculus
R. norvegicus
O. cuniculus
D. melanogaster
B. taurus
A. californica
A. americanum
L. donovani
C. elegans
X. laevis

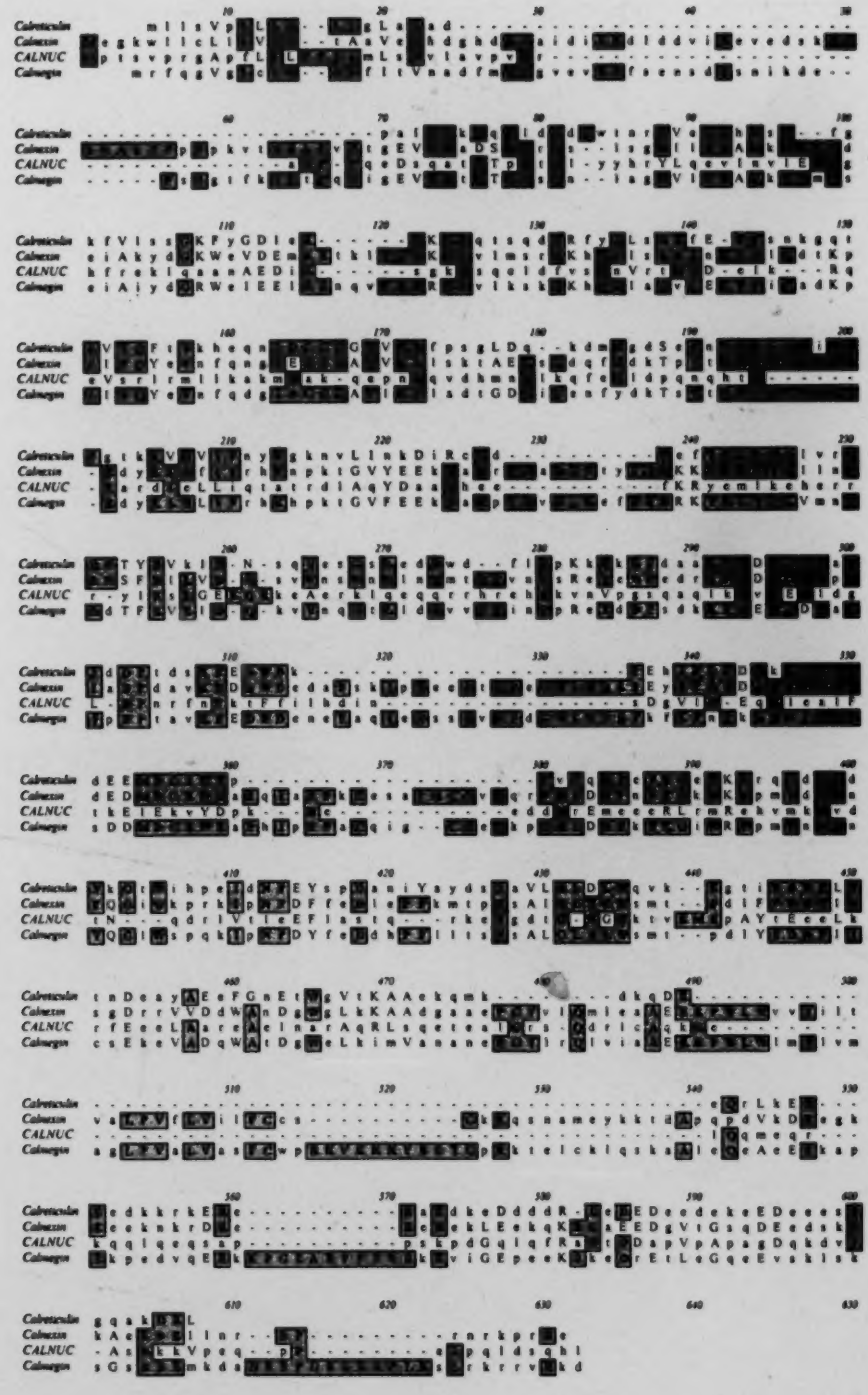
Calreticulin shares sequence similarity with a number of other Ca^{2+}-binding proteins (Figure 1-5). Calnexin was originally identified as having a high degree of sequence identity to calreticulin (Wada *et al.*, 1991). The luminal domain of calnexin shares several regions of extensive amino acid similarity with calreticulin, ranging from 42% to 78% identity. Several of the features that are most highly conserved in calreticulin are also present in calnexin. The highest degree of identity is located within a central segment, two of the three repeats found in calreticulin (KPEDWD) are found in calnexin, the third is interrupted by a novel sequence (Wada *et al.*, 1991). These repeat sequences contain a high affinity Ca^{2+}-binding site and also form the bulk of a lectin site that allow calreticulin and calnexin to specifically recognize monoglucosylated nascent proteins in the form of $Glc_1Man_9GlcNAc_2$. These repeat sequences have been shown to be critical for the lectin-like chaperone activity of both proteins (Vassilakos *et al.*, 1998).

Calreticulin also shares significant sequence homology with CALNUC, a major Ca^{2+}-binding, Golgi resident protein (Lin *et al.*, 1998). CALNUC was found to have 30% homology with rat calreticulin. The sequence similarity is found throughout the two proteins, however, most of homology is found in the P-domain (36%) and C-domain (43%) of the protein. The N-domain (26%) contains lower sequence similarity. The protein possesses both EF-hand motif consensus sequences and acidic regions, and due to its high homology to calreticulin it is suggested that it may play a key role in Ca^{2+} homeostasis in the *cis*-Golgi network (CGN) and *cis*-Golgi cisternae (Lin *et al.*, 1998).

Calmegin is a novel Ca^{2+}-binding protein that is specifically expressed during spermatogenesis. Calmegin has 30% homology to dog calnexin and partial homology to calreticulin within the repetitive internal sequences (Watanabe *et al.*, 1994). Calmegin is

Figure 1-5. Amino acid alignment of calreticulin, calnexin, calmegin and CALNUC

Comparison of *M. musculus* calreticulin, calnexin, CALNUC and calmegin amino acid sequences are shown. Identical residues are shown in bold uppercase with shading and outline, similarities are shown in uppercase, while mismatches are shown in lowercase.


Calreticulin
Calnexin
CALNUC
Calnexin

believed to function as a molecular chaperone during spermatogenesis (Ikawa *et al.*, 1997).

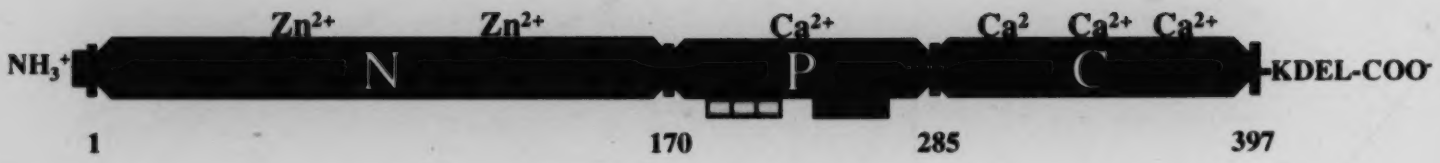
Domain structure of calreticulin

Structural predictions of calreticulin suggest that the protein has at least three structural and functional domains; an N-domain, P-domain and C-domain (Fig. 1-6 & Table 1-3) (Fliegel *et al.*, 1989a; Smith and Koch, 1989; Michalak *et al.*, 1992). The N-terminal part of the protein, encompassing the N- and P-domains of calreticulin, has the most conserved amino acid sequence. There is however variation seen within the acidic C-domain. The presence of a variant carboxy-terminal tail suggests divergent functions of this domain.

N-domain. The N-terminal half of the molecule has been named the N-domain and encompasses the first 170 amino acids of the protein. The N-terminal half of the molecule is predicted to be a highly folded, globular structure containing two short α-helices followed by 8 anti-parallel ß-strands connected by protein loops. The amino acid sequence of the N-domain is extremely conserved in all calreticulins, and is the most highly conserved region of the protein (Michalak, 1996). The N-domain binds Zn^{2+} (Heilmann *et al.*, 1993; Baksh *et al.*, 1995a; Baksh *et al.*, 1995b; Baksh *et al.*, 1995c). The N-domain interacts with the DNA binding domain of the glucocorticoid receptor (GR) *in vitro* (Burns *et al.*, 1994b), with Rubella virus RNA (Nakhasi *et al.*, 1994; Singh *et al.*, 1994; Atreya *et al.*, 1995), α-integrin (Rojiani *et al.*, 1991), and with PDI (Baksh *et al.*, 1995a) (Table 1-3). Interaction of this region of calreticulin with PDI inhibits PDI chaperone function (Baksh *et al.*, 1995a). The N-domain of calreticulin also inhibits

Figure 1-6. Domain structure of calreticulin

Calreticulin is divided into three structural and functional domains; N, P and C-domain. The N-domain is the N-terminal half of the molecule and the most conserved region of the protein. The P-domain comprises a proline-rich sequence and contains two sets of three, conserved amino acid repeats (shown in yellow and pink). The P-domain binds Ca^{2+} with high affinity and low capacity (K_D = 1 μM; B_{MAX} = 1 mole Ca^{2+}/mole protein). The C-terminal quarter of the protein, the C-domain, is highly acidic and negatively charged. This region binds Ca^{2+} with low affinity and high capacity (K_D = 2 nM; B_{MAX} = 25 moles Ca^{2+}/mole protein). The protein contains an N-terminal signal sequence and a C-terminal KDEL ER-retrieval signal. Ca^{2+} binding with high capacity to the C-domain and low capacity binding to the P-domain is shown. Zn^{2+} binding with high capacity to the N-domain is also shown.



Repeat A - PxxIxDPDAxKPEDWDE

Repeat B - GxWxPPxIxNPxYx

Signal sequence

Table 1-3: Functional properties of calreticulin domains

N-domain

Highly conserved amino acid sequence
Contains ER signal sequence
Interacts with PDI and inhibits PDI activity
Interacts with ERp57 and enhances ERp57 activity
Weak interactions with perforin
Binds Zn^{2+}
Potential phosphorylation site
Potential glycosylation site (bovine proteins)
Tumor- and peptide-specific immunity
Inhibits angiogenesis
Binds the DNA-binding domain of steroid receptors
Binds to α-subunit of integrin *in vitro*
Binds *Rubella* RNA

P-domain

Proline-rich domain
High-affinity Ca^{2+}-binding site
Lectin-like chaperone domain
Interacts with PDI
Interacts with perforin
Binds to a set of ER proteins
Amino acid sequence similarity to calnexin, calmegin and CALNUC
Putative glycosylation site (*Leishmania* protein)

C-domain

Rich in acidic amino acids
High-capacity Ca^{2+}-binding sites
Contains KDEL ER retrieval signal
Ca^{2+} "sensor" for calreticulin-protein interactions
Binds factor IX & X
Binds to a set of ER proteins
Anti-thrombotic activity
Binds to cell surface
Putative glycosylation site
Prevents retenosis

proliferation of endothelial cells and suppresses angiogenesis (Pike *et al.*, 1998). Calreticulin contains three Cys residues, all of which are located in the N-domain of the protein. Importantly, location of these amino acid residues is conserved in calreticulin ranging from higher plants to the human protein (Michalak *et al.*, 1992). Two of Cys residues found in the protein form a disulfide bridge (Cys^{88} and Cys^{120}) (Andrin *et al.*, 2000; Hojrup *et al.*, 2001), which may be important for proper folding and perhaps maintenance of structural integrity of the N-terminal region of calreticulin.

P-domain. The P-domain of the protein is located in the middle portion of the molecule and encompasses amino acids 187-285. The P-domain of calreticulin comprises a proline-rich sequence (hence the name) with three repeats of the 17 amino acid sequence PxxIxDPDAxKPEDWDE (Repeat A) followed by three repeats of the sequence GxWxPPxIxNPxYx (Repeat B). (Figure 1-6). This region of the protein binds Ca^{2+} with high affinity and the repeats may be essential for the high affinity Ca^{2+} binding of calreticulin (Baksh and Michalak, 1991; Tjoelker *et al.*, 1994). More importantly, repeats A and B are critical for the lectin-like chaperone activity of calreticulin (Vassilakos *et al.*, 1998). (Table 1-3). The P-domain of calreticulin interacts with PDI (Baksh *et al.*, 1995a) and perforin (Andrin *et al.*, 1998; Fraser *et al.*, 1998), a component of the cytotoxic T-cell granules. The P-domain is one of the most interesting and unique regions of the protein because of its lectin-like activity and amino acid sequence similarities to other Ca^{2+} binding chaperones. Studies on the nuclear magnetic resonance (NMR) spectroscopy structure of calreticulins P-domain have recently provided some insight into its structure. These studies indicate that the NMR structure of calreticulin (P-domain) is characterized by an extended hairpin fold. The N- and C-termani of calreticulins P-

domain are in close proximity and suggest that in the native protein the P-domain constitutes a protrusion from the calreticulin core (Ellgaard *et al.*, 2001a; Ellgaard *et al.*, 2001b).

C-domain. The C-terminal region of the protein (C-domain) includes amino acids 286-401 and is the most variant region of the protein. The C-domain is highly acidic and negatively charged, and terminates with the KDEL ER retrieval sequence (Fliegel *et al.*, 1989a; Smith and Koch, 1989). (Table 1-3). This domain of the protein is responsible for the low affinity, high capacity Ca^{2+}-binding activity of the protein (Baksh and Michalak, 1991). It binds to blood clotting factors, specifically the vitamin K-dependent coagulation factors - Factor IX, X and prothrombin (Kuwabara *et al.*, 1995) and inhibits injury-induced restenosis (Dai *et al.*, 1997).

Glycosylation of calreticulin

There is still much speculation as to whether or not calreticulin is glycosylated. Calreticulin contains a putative N-linked glycosylation site at amino acid 327 that has been conserved in calreticulin from a diverse range of species (Michalak *et al.*, 1992). This conservation over millions of years would suggest that it may be relevant to the proteins function. However, the glycosylation pattern of calreticulin is heterogeneous and does not appear to be a conserved property of the protein. No glycosylation of calreticulin has been detected in skeletal or smooth muscle (Milner *et al.*, 1991), human (McCauliffe *et al.*, 1990a), mouse (Lewis *et al.*, 1985), chicken or rabbit liver (Waisman *et al.*, 1985). In contrast to this, calreticulin isolated from bovine liver and brain (Matsuoka *et al.*, 1994), rat liver (Van *et al.*, 1989), Chinese Hamster Ovary (CHO) cell (Jethmalani and

Henle, 1994) and *Leichmania* calreticulin (Joshi *et al.*, 1996) have been reported to be glycosylated. One possibility is that these apparent contradictions are due to species and tissue specific differences in glycosylation. In agreement with this, cells exposed to heat stress have been documented to undergo a process known as 'prompt protein glycosylation' whereby a specific set of proteins is rapidly glycosylated after heat stress, while constitutive protein glycosylation ceases (Henle *et al.*, 1993). Calreticulin is glycosylated in CHO cells exposed to heat stress (Henle *et al.*, 1993) and upon amino acid deprivation in CHO cells (Heal and McGivan, 1998). Perhaps glycosylation can prevent aggregation of key cellular proteins under certain stressful conditions, thus ensuring cell survival. Many heat shock proteins are molecular chaperones, therefore their glycosylation (prompt stress glycoproteins) may allow the development of thermo-tolerance and hence cell survival.

Nucleotide binding to calreticulin

Although there is no direct evidence that calreticulin can bind ATP, there are numerous factors that suggest this possibility. Calreticulin and other ER chaperones (GRP170, PDI, BiP, ERp72 and GRP94) form Ca^{2+}-dependent and ATP-sensitive complexes with misfolded proteins (Nigam *et al.*, 1994; Dierks *et al.*, 1996). In addition, ATP was found to enhance calreticulins ability to suppress aggregation of non-glycoproteins, whereas engagement of its lectin site with purified oligosaccharide abolishes this function (Saito *et al.*, 1999). Oligosaccharide binding to calreticulin is only slightly enhanced in the presence of high concentrations of adenosine nucleotides (Vassilakos *et al.*, 1998). Calnexin, the membrane-spanning homologue of calreticulin,

binds Mg^{2+}-ATP in its luminal domain, and binding of ATP induces conformational changes in the protein (Ou *et al.*, 1995). Calnexin binding Mg^{2+}-ATP induced calnexin to be converted from a monomeric to an oligomeric form that coincided with markedly increased proteinase sensitivity (Ou *et al.*, 1995). A more recent study demonstrated that calreticulin isolated from canine tissues contained an endogenous phosphate (Cala, 1999). Analysis of calreticulin tryptic peptides localized the phosphate binding to the C-terminal region of the protein. However, this putative phosphate does not appear to results from phosphorylation as it was tightly associated with a peptide that did not contain any phosphorylatable residues (Cala, 1999). However, binding of ATP or any other nucleotide to calreticulin has yet to be demonstrated.

Cation binding to calreticulin

The ability of calreticulin to bind Ca^{2+} has been established by many laboratories (Ostwald and MacLennan, 1974; Waisman *et al.*, 1985; Van *et al.*, 1989; Treves *et al.*, 1990; Baksh and Michalak, 1991; Milner *et al.*, 1991). Calreticulin contains two Ca^{2+}-binding sites (Baksh and Michalak, 1991; Ostwald and MacLennan, 1974). The protein binds Ca^{2+} with both high affinity and low capacity, as well as with low affinity and high capacity. Calreticulin does not contain the consensus EF hand motif (Fliegel *et al.*, 1989a; Smith and Koch, 1989), characteristic of other high affinity Ca^{2+}-binding proteins, such as calmodulin or tropinin C (Kretsinger, 1988). The low affinity, high capacity Ca^{2+}-binding sites are localized to the C-domain of calreticulin, whereas the high affinity, low capacity Ca^{2+}-binding site is located within the P-domain of the protein (Baksh and Michalak, 1991). What is the role of Ca^{2+} binding to calreticulin? The most logical

function of Ca^{2+} binding to calreticulin is for storage. In this way calreticulin would provide cells with a 'reservoir' of intracellular Ca^{2+} to carry out a wide variety of Ca^{2+}-dependent processes without having to rely on the availability of extracellular Ca^{2+}. Is Ca^{2+}-binding to calreticulin critical for the many other functions proposed for the protein? Interactions between calreticulin and denatured polypeptides may be Ca^{2+}-dependent (Nigam *et al.*, 1994). Ca^{2+} binding to the P-domain of calreticulin has been shown to be essential for oligosaccharide binding (Vassilakos *et al.*, 1998).

Ca^{2+} binding to the low affinity, high capacity site of calreticulin is reduced in the presence of millimolar concentrations of Mg^{2+}, indicating that the protein also binds Mg^{2+} with relatively low affinity *in vitro* (Baksh and Michalak, 1991).

Calreticulin has also been demonstrated to bind Zn^{2+}. Calreticulin binds Zn^{2+} with relatively low affinity (B_{max} = 14 moles Zn^{2+}/ mole of protein; K_D = 300 μM) (Khanna *et al.*, 1986; Khanna *et al.*, 1987). Upon binding of Zn^{2+}, calreticulin was found to undergo dramatic conformational changes as measured by changes in intrinsic fluorescence and its circular dichroism (CD) spectrum (Khanna *et al.*, 1986; Khanna *et al.*, 1987). This appears to be in contrast to Ca^{2+} binding to calreticulin, which does not appear to induce any dramatic conformational changes in calreticulin structure (Ostwald and MacLennan, 1974; Khanna *et al.*, 1986; Van *et al.*, 1989). Zn^{2+} binding also causes an increase in hydrophobicity of calreticulin as monitored by intrinsic protein fluorescence, binding of the hydrophobic fluorescent probe 8-anilino-1-naphthalenesulphonate (ANS), and also its Zn^{2+}-dependent interaction with the hydrophobic matrix of phenyl-Sepharose (Khanna *et al.*, 1986; Khanna *et al.*, 1987; Heilmann *et al.*, 1993). Calreticulin does not contain any consensus amino acid sequences for a 'Zn^{2+}-finger' motif, known to be associated with

Zn^{2+}-binding sites in proteins (Berg, 1990; Vallee and Auld, 1990). It is well documented however, that multiple Cys and histidine (His) residues can bind Zn^{2+} (Berg, 1990; Vallee and Auld, 1990). Diethyl pyrocarbonate (DEPC) modification studies by Baksh *et al.* (Baksh *et al.*, 1995b) revealed that five out of the seven His residues in the N-domain of calreticulin may be involved in Zn^{2+} binding to the protein (Baksh *et al.*, 1995b). It is possible that calreticulin may play a role in the regulation of intracellular levels of Zn^{2+}. More recent studies indicate that Zn^{2+} binding to calreticulin may play a role in the regulation of calreticulin function. Studies by Saito *et al* demonstrated that Zn^{2+} enhances calreticulins ability to suppress aggregation of non-glycoproteins (Saito *et al.*, 1999).

Tissue distribution and cellular localization of calreticulin

Calreticulin is a ubiquitous protein that has been found in a wide variety of cell types in a wide variety of species from mammals (Michalak, 1996) to plant cells (Menegazzi *et al.*, 1993; Opas *et al.*, 1996b). Calreticulin has also been found in many different tissue types (Khanna and Waisman, 1986; Treves *et al.*, 1990; Fliegel *et al.*, 1989b). The protein is most abundant in pancreas, liver and testis – tissues that are involved in the production of high amounts of protein (Khanna and Waisman, 1986), with protein levels in these tissues ranging from 200 – 550 μg/g of tissue. Lower quantities of calreticulin are found in the kidney, spleen, adrenal cortex and parathyroid, which have approximately 100 μg/g tissue (Khanna and Waisman, 1986). The cerebral cortex and muscle tissues have the lowest amounts of calreticulin (~ 20 μg/g tissue) (Khanna and Waisman, 1986).

Since its initial identification as an ER luminal protein, numerous studies have confirmed this localization in many diverse species including plants (Fliegel *et al.*, 1989b; Michalak *et al.*, 1991;Milner *et al.*, 1991; Opas *et al.*, 1991; Chen *et al.*, 1994; Bastianutto *et al.*, 1995; Michalak *et al.*, 1996; Mery *et al.*, 1996; Opas *et al.*, 1996a; Tharin *et al.*, 1996; Burns *et al.*, 1997; Crofts and Denecke, 1998; Fadel *et al.*, 1999; John *et al.*, 1998; Mesaeli *et al.*, 1999). It is not surprising that calreticulin is localized to the ER, as the protein contains an N-terminal ER signal sequence, ensuring its initial targeting to the ER membranes, and terminates with a KDEL retention signal (Fliegel *et al.*, 1989a). Using deletion mutants of calreticulin, Sönnichsen *et al* (Sonnichsen *et al.*, 1994) showed that the KDEL retrieval signal and the high density of negative residues adjacent to the KDEL sequence were required for retention of calreticulin in the ER. These results suggest that there are two independently operating retention/retrieval mechanisms for calreticulin: one providing for a KDEL-based retrieval system for escaped calreticulin present in the Golgi apparatus; the other a direct retention in the ER with very high capacity and having Ca^{2+}-dependent properties (Sonnichsen *et al.*, 1994). It is not inconceivable that the 'Ca^{2+}-matrix' described by Koch and Sambrook is responsible for this retention (Koch, 1987; Sambrook, 1990).

Surprisingly, there is still considerable controversy in the literature concerning the other cellular localizations of the protein. This is likely because of the difficulties to explain the role of calreticulin in control of steroid-sensitive gene expression, cell adhesion and other extra-ER functions (Burns *et al.*, 1994a; Burns *et al.*, 1994b; Dedhar, 1994; Dedhar *et al.*, 1994; Leung-Hagesteijn *et al.*, 1994; Coppolino *et al.*, 1995; St-Arnaud *et al.*, 1995; Wheeler *et al.*, 1995; Desai *et al.*, 1996; Michalak *et al.*, 1996; Opas

et al., 1996a; St-Arnaud *et al.*, 1996; Burns *et al.*, 1997; Coppolino *et al.*, 1997; Krause and Michalak, 1997; Coppolino and Dedhar, 1998; Sela-Brown *et al.*, 1998; Fadel *et al.*, 1999; Perrone *et al.*, 1999). It was initially proposed that these effects might be due to the direct interaction between calreticulin and the DBD of steroid receptors (Burns *et al.*, 1994b; Dedhar *et al.*, 1994) and the cytoplasmic tail of α-integrin (Rojiani *et al.*, 1991; Dedhar *et al.*, 1994; Coppolino and Dedhar, 1998). For calreticulin to bind to these molecules the protein would have to be present in the nucleus and/or cytosol. Calreticulin-like immunoreactivity was detected in the nucleus of some cells (Opas *et al.*, 1991; Dedhar *et al.*, 1994), such as squamous carcinoma cell nuclei in response to ionizing radiation (Ramsamooj *et al.*, 1995) or in the nucleus of the dexamethasone-treated LM (TK^-) cells (Roderick *et al.*, 1997). However, studies indicate that calreticulin is not a nuclear resident protein and earlier identification of the protein in the nucleus (Opas *et al.*, 1991) was likely an artifact of immunostaining (Michalak *et al.*, 1996). The proposed presence of calreticulin in the cytosol has recently been described, where it functions as a receptor for nuclear protein export (Holaska *et al.*, 2001). However, due to the lack of firm experimental evidence localizing calreticulin to the cytoplasm in this work and others, more work must be conducted to solve this and other issues (Holaska *et al.*, 2001). The likelihood of calreticulin acting as a signaling molecule from the ER seems more likely.

The protein has also been localized to many other cellular locations, where it performs a diverse range of biological functions. A Ca^{2+}-binding protein was isolated from rat spermatogenic cells (Nakamura *et al.*, 1991), which was later identified as calreticulin (Nakamura *et al.*, 1993). Immuno-histochemical analysis and

immunoelectron microscopy revealed that the protein is localized primarily to the acrosome of spermatids and mature sperm and was also found in the Golgi apparatus (Nakamura *et al.*, 1993). It was proposed that calreticulin may be incorporated into the acrosomal vesicle *via* the Golgi apparatus, during spermatogenesis, and may play an important role in the regulation of cell functions such as sperm motility and acrosome reaction. The presence of both Ca^{2+} (Yanagimachi and Usui, 1974) and Zn^{2+} (Clapper *et al.*, 1985) was shown to be important for sperm-egg fusion to occur – calreticulin may be the source of both ions. This calreticulin also contained a cleavable N-terminal signal sequence and a KDEL ER retrieval signal. The protein somehow eludes the ER retention/retrieval mechanism and escapes into these granules (Nakamura *et al.*, 1993). The mechanism by which it achieves this remains to be determined.

Calreticulin has also been localized to the cytoplasmic granules of the cytotoxic T-lymphocytes (CTLs) (Dupuis *et al.*, 1993). Low levels of calreticulin mRNA and protein can be found in resting, mouse and human CTLs (Burns *et al.*, 1992). Calreticulin expression is unregulated in stimulated T-cells as measured by an induction of calreticulin mRNA and a five-fold increase in calreticulin protein levels (Burns *et al.*, 1992). Later, it was shown that not only was calreticulin a component of T-cell lytic granules, but that it also associates with perforin (Dupuis *et al.*, 1993). Perforin is involved in the killing function of CTLs by forming toxic pores in the target cell membrane and requires Ca^{2+} to become activated (Gardner, 1989). Since perforin is activated and becomes lytic in the presence of Ca^{2+} it was proposed that the role of calreticulin may be to prevent organelle autolysis due to its Ca^{2+} chelation capacity (Dupuis *et al.*, 1993) . Later studies demonstrated that perforin interacts strongly with the

P-domain of calreticulin, and that this association is Ca^{2+}-dependent (Andrin *et al.*, 1998). Perforin binds to calreticulin in the absence of Ca^{2+} and the two proteins dissociate upon exposure to 100 μM or higher Ca^{2+} concentration (Andrin *et al.*, 1998). It was proposed that calreticulin may act as a Ca^{2+}-regulated chaperone for perforin, serving to protect the T-cell during the biogenesis of granules and regulating perforin lytic action after release (Andrin *et al.*, 1998). Later work by Fraser *et al* (Fraser *et al.*, 1998) demonstrated that 10^{-7} M calreticulin blocked perforin-mediated lysis in the hemolytic model system using erythrocytes as targets. They observed Ca^{2+}-dependent binding of calreticulin to erythrocyte membranes, and proposed that membrane-bound calreticulin may prevent hydrophobic entry of perforin into membranes and/or prevent perforin from assembling into polyperforin pores (Fraser *et al.*, 1998).

Calreticulin is also found in numerous extracellular locations implying that it may be secreted from cells. There are a number of possible explanations for how calreticulin gets to the cell surface. Isoforms of calreticulin may be synthesized that do not contain a KDEL sequence. In support of this is the presence of calreticulin on the surface of cells that is not recognized by antibodies against KDEL (Xiao *et al.*, 1999). The isolation of a modified form of calreticulin lacking the C-terminal hexapeptide, including the KDEL sequence, from human placenta was recently described (Hojrup *et al.*, 2001). Another possibility is that binding of another molecule masks the KDEL sequence. There have been examples where calreticulin was shown on the cell surface in complex with the KDEL receptor (Zhu *et al.*, 1997). Another possibility is saturation of the ER retention machinery (Crofts *et al.*, 1999). Whatever the mechanism utilized, it is clear that calreticulin, an ER resident protein, is found in a number of extracellular locations where

it causes/mediates many effects. A small percentage of calreticulin has been found in human serum (~ 60 ng/ml), supporting the potential for the generation of circulating antibodies to calreticulin observed in a variety of autoimmune diseases (Sueyoshi *et al.*, 1991). The protein has also been found on the surface of activated T-cells in association with unfolded major histocompatibility complex (MHC) class I molecules, the folding and assembly of which is facilitated by calreticulin (Arosa *et al.*, 1999). The protein has been identified on the surface of red blood cells, along with a number of ER proteins (BiP, PDI) in patients with congenital dyserythropoietic anemia (Alloisio *et al.*, 1996). It has also been found to elicit tumor- and peptide-specific immunity, a fact attributed to the peptides associated with calreticulin and not to the calreticulin itself *per se* (Basu and Srivastava, 1999). Calreticulin has also been demonstrated to bind to glycosylated laminin on the surface of B16 mouse melanoma cells (White *et al.*, 1995), which may be responsible for the proteins ability to trigger cell spreading (White *et al.*, 1995). It interacts with glycosylated laminin in a Ca^{2+}-dependent manner (McDonnell *et al.*, 1996). It has also been reported that cell surface calreticulin can interact with integrins (Zhu *et al.*, 1997; Kwon *et al.*, 2000). Calreticulin, or fragments of calreticulin, were found to inhibit proliferation of endothelial cells, suppress angiogenesis, and inhibit tumor growth (Pike *et al.*, 1998; Pike *et al.*, 1999). It is present in the saliva of a variety of ticks including *Amblyomma americanum L* and *Ixodes scapularis* although its role is unknown (Sanders *et al.*, 1998; Sanders *et al.*, 1999).

Calreticulin is released from activated neutrophils and can bind to C1q, the first subcomponent of complement (Eggleton *et al.*, 1994; Kishore *et al.*, 1997). Both the N- and P-domains of calreticulin were found to interact with C1q, and through this

interaction may be able to interfere with some of the functions of the complement pathway (Kishore *et al.*, 1997). The cell surface receptor C1q-R, a homologue of calreticulin was also shown to bind C1q (Eggleton *et al.*, 1994). In addition, evidence for the presence of calreticulin in the extracellular environment comes from the ability of calreticulin to act as an autoantigen in patients with a variety of autoimmune diseases. Autoantibodies to calreticulin have been found in ~40-60 % of patients with SLE (Eggleton *et al.*, 1997; Kovacs *et al.*, 1998) and also in a number of patients with secondary Sjögrens syndrome (Eggleton *et al.*, 2000). The N-terminal half of calreticulin was found to be more antigenic (69% of SLE sera from active disease patients), while the C-domain was not found to be antigenic (Eggleton *et al.*, 2000). In some autoimmune diseases such as SLE, a major contributing factor is the failure to clear immune complexes, a process that is largely mediated by C1q. This fact is highlighted by the fact that patients who lack C1q almost always develop active SLE (Walport *et al.*, 1998). Binding of calreticulin to C1q may inhibit its ability to clear immune complexes, therefore contributing to the disease state overall. Antibodies to calreticulin have also been detected in a variety of other autoimmune diseases, including rheumatoid arthritis (Verreck *et al.*, 1995), celiac disease (Tuckova *et al.*, 1997), halothane hepatitis (Gut *et al.*, 1993) and complete congenital heart block (Orth *et al.*, 1996).

Calreticulin deficient mouse

Major insights into the functions of calreticulin have come from the development of a calreticulin deficient mouse and from derived calreticulin-deficient cell lines (Mesaeli *et al.*, 1999; Nakamura *et al.*, 2000). The calreticulin gene disruption is the first

reported knock out for a gene encoding an ER luminal protein. Since calreticulin is involved in a number of diverse and important functions, it was anticipated that the protein would be essential for organism survival. This was indeed found to be the case, as the $crt^{-/-}$ embryos were only viable until 14.5 days into gestation (Mesaeli *et al.*, 1999; Rauch *et al.*, 2000). The most obvious defect in the embryos was in the heart, with cardiac failure most likely contributing to embryonic lethality, indicating that calreticulin must play a role in cardiac development (Mesaeli *et al.*, 1999). Calreticulin is expressed at low levels in the mature heart, however calreticulin expression is up-regulated in the developing heart (Mesaeli *et al.*, 1999). Recent work by Guo *et al* (Guo *et al.*, 2001) indicates that the cardiac transcription factor Nkk2.5 activates expression of calreticulin in the heart, while the transcription factor COUP-TF1 may mediate its postnatal suppression (Guo *et al.*, 2001). It is evident from the calreticulin gene-disruption studies that calreticulin is important in the developing heart – but which of the many functions of calreticulin is responsible for mediating this role? The pathology displayed in calreticulin-deficient mice may relate to a role of calreticulin in the modulation of Ca^{2+} homeostasis or to its role as a chaperone. Studies by Nakamura *et al* (Nakamura, 2001) have shown that calreticulin-deficient mouse embryonic fibroblasts (MEF) have a lower capacity for Ca^{2+} storage, although the free $[Ca^{2+}]_{ER}$ remained unchanged. Results from this work also indicate that calreticulin plays a role as a chaperone of the bradykinin receptor. Bradykinin-induced Ca^{2+} release was impaired in these cells, which could be restored by expression of full-length calreticulin in these cells (Nakamura, 2001). This is the first direct evidence *in vivo*, that the C-domain of calreticulin plays an important role in Ca^{2+} storage and that the N-domain functions as a chaperone. The generation of $crt^{-/-}$

fibroblasts provides an important tool for the investigation of the exact role that calreticulin plays in it's many proposed functions.

Functions of calreticulin

Calreticulin has been implicated to participate in numerous cellular functions (Table 1-4, (Burns *et al.*, 1994b; Sontheimer *et al.*, 1995; Michalak *et al.*, 1996; Eggleton *et al.*, 1997; Helenius *et al.*, 1997; Krause and Michalak, 1997; Coppolino and Dedhar, 1998; Crofts and Denecke, 1998; Nakhasi *et al.*, 1998). This strongly exemplifies the central role that the ER plays in a variety of cellular functions. It is not surprising, therefore that changes in calreticulin expression and function has profound effects on many cellular functions. However, there is a widespread agreement that calreticulin serves two major functions in the ER lumen: (i) as a chaperone and (ii) as a regulator of Ca^{2+} homeostasis.

Calreticulin and chaperone function

Molecular chaperones prevent the aggregation of partially folded proteins, increase the yield of correctly folded proteins and assembly, and also increase the rate of correctly folded intermediates by recruiting other folding enzymes. It is well established that calnexin functions as a molecular chaperone for newly synthesized glycoproteins (Ou *et al.*, 1993; Bergeron *et al.*, 1994; Hammond and Helenius, 1994; Rajagopalan and Brenner, 1994; Rajagopalan *et al.*, 1994; Tatu and Helenius, 1997; Tector *et al.*, 1997; Zapun *et al.*, 1997). The sequence similarity between calreticulin and calnexin led

Table 1-4: Selected functions attributed to calreticulin

ER functions

Chaperone function

Lectin-like Function
Essential for glycoprotein maturation
Important for MHC class I assembly
Endoplasmic Reticulum Chaperone
for HIV Type 1 Envelope Glycoprotein
Chaperone for Myeloperoxidase
Quality Control in the ER

Ion Binding and Storage

Ca^{2+} binding & storage
Zn^{2+} binding & storage
- Modulates Ins-P_3-dependent Ca^{2+}-release
Modulates SERCA2b function
- Regulation of store-operated Ca^{2+} influx

Other functions

Adhesion

Activates integrins
Inhibits angiogenesis
Initiates cell spreading
Acrosome function & sperm motility

Cytotoxic T-cell function

Interacts with perforin
Inhibits perforin-dependent killing
Component of lytic granules in CTL and NK

Gene expression

Control of Rubella virus replication
Control of steroid sensitive gene expression
Regulates expression of vinculin

Blood function

Antithrombotic activity
Binds C1q Complement
Component of tick saliva
Modulates platelet activation

Others

Autoantigen in a variety of auto-immune diseases
Affects neuronal development
Affects cardiac development
Increases sensitivity to apoptosis
Modulates nuclear import & export
Stress protein
Wound healing

researchers to postulate that calreticulin may also serve to function as a molecular chaperone. In the last few years there have been numerous publications documenting that calreticulin functions as a lectin-like molecular chaperone for many proteins (Kim and Arvan, 1995; Halaban *et al.*, 1997; Hebert *et al.*, 1997; Kuznetsov *et al.*, 1997; Tatu and Helenius, 1997; Wada *et al.*, 1997; Bass *et al.*, 1998; Crofts and Denecke, 1998; Harris *et al.*, 1998; Nauseef *et al.*, 1998; Pipe *et al.*, 1998; Labriola *et al.*, 1999; Vassilakos *et al.*, 1998; Zapun *et al.*, 1998). Use of glucosidase inhibitors established that interaction of calreticulin with newly synthesized glycoproteins was dependent on the monoglucosylated form of the carbohydrate chain (Wada *et al.*, 1995; Otteken and Moss, 1996; Peterson *et al.*, 1996; Zhang *et al.*, 1997). This is similar to the binding properties of calnexin (Bergeron *et al.*, 1994; Allen and Bulleid, 1997; Hebert *et al.*, 1995). Both bind to $Glc_1Man_9GlcNAc_2$ oligosaccharides and recognize the terminal glucose and four internal mannose moieties (Spiro *et al.*, 1996; Vassilakos *et al.*, 1998). Both chaperones are highly versatile being involved in the "quality control" process during the synthesis of a variety of molecules including ion channels, surface receptors, integrins and transporters (Helenius *et al.*, 1997). Although, these chaperones can be considered two of the most important molecules pres[redacted]in a cell we still do not fully comprehend how calreticulin assists in protein folding, how it acts jointly with other ER chaperones, and what role ER Ca^{2+} plays in these processes.

Calreticulin and calnexin appear to work in synchrony based on the fact that ternary complexes between calreticulin, calnexin and substrate have been identified (Otteken and Moss, 1996; Tatu and Helenius, 1997). Calnexin and calreticulin share many substrates, and may form a link of lectin-like chaperones transferring glycoproteins

from one to the other to ensure proper folding (Nauseef *et al.*, 1995; Wada *et al.*, 1995; Hebert *et al.*, 1996; Rodan *et al.*, 1996; Van Leeuwen and Kearse, 1996a; Van Leeuwen and Kearse, 1996c; Helenius *et al.*, 1997; Tatu and Helenius, 1997; Bass *et al.*, 1998; Choukhi *et al.*, 1998; Pipe *et al.*, 1998; Zhang and Salter, 1998; Veijola and Pettersson, 1999). Do calreticulin and calnexin depend on each other for proper chaperoning? The MHC class I complex is an excellent example of a molecule that employs both calreticulin and calnexin for proper folding (Degen and Williams, 1991; Margolese *et al.*, 1993; Ware *et al.*, 1995; Williams, 1995; Sadasivan *et al.*, 1996; Suh *et al.*, 1996; Elliott *et al.*, 1997; Zhang *et al.*, 1997; Harris *et al.*, 1998; Zhang and Salter, 1998). MHC class I molecules consist of two non-covalently linked subunits - an integral membrane glycoprotein (α chain), a small soluble protein ß2-microglobulin (ß2-m), and a peptide of 8-11 residues. Calnexin and calreticulin facilitate the folding of MHC class I α chains (Degen and Williams, 1991; Margolese *et al.*, 1993; Ware *et al.*, 1995; Williams, 1995; Sadasivan *et al.*, 1996; Suh *et al.*, 1996; Elliott *et al.*, 1997; Zhang *et al.*, 1997; Harris *et al.*, 1998; Zhang and Salter, 1998). In mouse and human, α chains associate with calnexin soon after synthesis *via* interactions with both immature glycans and with residues in the transmembrane domain of α chains (York and Rock, 1996). In humans, ß2-m binding to α chain displaces calnexin and the resulting α chain-ß2-m heterodimer binds calreticulin (Sadasivan *et al.*, 1996; Suh *et al.*, 1996). MHC class I expression and transport to the cell surface are not changed in calnexin deficient cells (Balow *et al.*, 1995; Sadasivan *et al.*, 1995; Scott and Dawson, 1995; Sadasivan *et al.*, 1996; Prasad *et al.*, 1998) suggesting that calnexin is not essential for MHC class I synthesis and transport. Although indirectly, these findings indicate that calreticulin can function in the

absence of calnexin, and that MHC class I processing can occur without calnexin. MHC class I synthesis and trafficking has only recently been investigated in calreticulin deficient cells (Gao, 2001). Assembly of α chain with ß2-m occurs normally in calreticulin-deficient cells, but subsequent loading with optimal, stabilizing peptides is defective. This defect in peptide loading was not complete, but resulted in a ~ 50-80% loss compared to when calreticulin was present. Also these peptides were able to escape ER QC and were released into the secretory pathway. This defect in peptide loading appears to be specific for calreticulin, as it could be rectified by transfection of calreticulin to the cells, but not of a soluble form calnexin (Gao, 2001). These results demonstrate that unlike calnexin, calreticulin is essential for MHC class I synthesis and transport, being specifically involved in peptide loading.

Given the identical lectin specificities of calreticulin and calnexin, it is not surprising that there is an overlap in the glycoproteins bound. Although many glycoproteins bind to both calreticulin and calnexin, there are glycoproteins specifically bound by only one of the chaperones (Wada *et al.*, 1995; Hebert *et al.*, 1996; Otteken and Moss, 1996; Nauseef *et al.*, 1998; Pipe *et al.*, 1998). This raises the question as to whether each has distinct functions that are utilized at different stages in glycoprotein synthesis. Alternatively, they may be functionally interchangeable, and binding to different glycoproteins may occur due to differences in topological environment (membrane-bound versus soluble) or differences in polypeptide binding specificity. Danilczyk *et al* sought to address this question and demonstrated that the topological environment can explain differences in glycoproteins bound by calreticulin and calnexin (Danilczyk *et al.*, 2000). Membrane-bound calreticulin binds a similar set of

glycoproteins as calnexin, while a soluble form of calnexin (residues 1-387) binds a pattern of glycoproteins that resemble calreticulin (Danilczyk *et al.*, 2000).

Using assays performed to characterize the functions of the molecular chaperones HSP90, HSP70 and HSP60, Saito *et al* demonstrated that in addition to its lectin-like function, calreticulin can also behave as a true molecular chaperone. Calreticulin was found to distinguish between native and non-native conformations of non-glycosylated substrates and did not recognize native proteins (Saito *et al.*, 1999). A 'dual-binding model' was proposed whereby calreticulin, in addition to interacting with carbohydrates and thereby functioning as a lectin-like chaperone, can also bind exposed hydrophobic polypeptide sequences on nascent proteins and thus also serve as a true molecular chaperone (Saito *et al.*, 1999). A recent study has characterized the polypeptide-binding properties of calreticulin with denatured ovalbumin (Jorgensen *et al.*, 2000).

Calreticulin – regulation of Ca^{2+} homeostasis

One way by which a cell can maintain intracellular Ca^{2+} levels is through Ca^{2+}-binding proteins that buffer the Ca^{2+} content within the cell and within cellular organelles. Ca^{2+}-binding chaperones augments the Ca^{2+} storage capacity of the ER lumen. Calreticulin has been found to play an important role in Ca^{2+} homeostasis in a variety of cells (Liu *et al.*, 1994; Camacho and Lechleiter, 1995; Mery *et al.*, 1996; Coppolino *et al.*, 1997; John *et al.*, 1998). Overexpression of the protein in a variety of cellular systems (Bastianutto *et al.*, 1995; Mery *et al.*, 1996; Opas *et al.*, 1996a), does not have a significant effect on the $[Ca^{2+}]_C$, however, it does result in an increased amount of intracellularly stored Ca^{2+} (Bastianutto *et al.*, 1995; Mery *et al.*, 1996; Michalak *et al.*,

1996). This is due to the increased Ca^{2+} binding to the high capacity Ca^{2+}-binding region in the C-domain of calreticulin. Studies by Nakamura *et al* demonstrated that in calreticulin-deficient MEF, the ER has a lower capacity for Ca^{2+} storage (Nakamura, 2001). By transfection of these calreticulin-deficient cells with expression vectors encoding different domains of the protein it was shown that Ca^{2+}-binding to the C-domain of calreticulin *in vivo* is responsible for determination of the ER Ca^{2+}-storage capacity. It appears therefore that calreticulin simply binds more Ca^{2+} in the ER lumen, as the level of the free $[Ca^{2+}]_{ER}$, as measured by an ER-targeted 'cameleon' reporter remained unchanged (Nakamura, 2001). Studies using anti-sense nucleotides demonstrated that decreasing the levels of calreticulin in cells led to a concomitant decrease in the Ca^{2+} storage capacity of the $InsP_3$ -sensitive Ca^{2+} pool (Liu *et al.*, 1994). Interestingly, Ca^{2+} storage capacity of the ER is not changed in the calreticulin deficient embryonic stem (ES) or MEF cells (Coppolino *et al.*, 1997; Mesaeli *et al.*, 1999). This is likely because other ER luminal Ca^{2+} binding chaperones (GRP94, BiP, and PDI) compensate for the loss of calreticulin.

- **Calreticulin, an ER luminal regulator of SERCA function**

Ca^{2+} is transported into the ER lumen by SERCA (Pozzan *et al.*, 1994). There are three differentially expressed genes encoding the SERCA protein (MacLennan *et al.*, 1997; Wu *et al.*, 1997). SERCA1a and 1b are expressed in fast-twitch skeletal muscle, while SERCA3 is expressed in a limited set of non-muscle cells (MacLennan *et al.*, 1997; Wu *et al.*, 1997). SERCA2a is the cardiac/slow-twitch muscle isoform, whereas SERCA2b, with a C-terminal extension, is expressed in smooth muscle and non-muscle

tissues that also contain high levels of calreticulin (Lytton *et al.*, 1989; Krause *et al.*, 1990; Lytton *et al.*, 1991; Arber *et al.*, 1992; Lytton *et al.*, 1992; Van Delden *et al.*, 1992; Enyedi *et al.*, 1993; Johnson *et al.*, 1993; Stendahl *et al.*, 1994; Michalak, 1996; MacLennan *et al.*, 1997; Vanlingen *et al.*, 1997; Wu *et al.*, 1997; John *et al.*, 1998; Majeed *et al.*, 1999).

Does calreticulin affect the function of SERCA? Camacho's group carried out elegant studies on the role of calreticulin in Ca^{2+} homeostasis utilizing the *Xenopus* oocyte model (Camacho and Lechleiter, 1995; John *et al.*, 1998). They demonstrated that co-expression of calreticulin with SERCA2b (but not SERCA1 or 2a) results in a sustained elevation in Ca^{2+} release without concomitant oscillations upon injection of $InsP_3$ (Camacho and Lechleiter, 1995; John *et al.*, 1998). SERCA2b has an additional trans-membrane segment and a C-terminal 12 residue tail localized to the ER lumen (Lytton *et al.*, 1989; Lytton *et al.*, 1991;Campbell *et al.*, 1992; Lytton *et al.*, 1992; Verboomen *et al.*, 1994). It contains a putative N-glycosylation site (residue N1036) (Lytton *et al.*, 1989; Lytton *et al.*, 1991; Lytton *et al.*, 1992). Site-directed mutagenesis of the Asn1036 (John *et al.*, 1998) or truncation studies involving Asn1036 (Verboomen *et al.*, 1994) revealed that this residue is critical for calreticulin-dependent effects on SERCA2b function and for its isoform specific functional differences. Effects of calreticulin on SERCA2b involve the P-domain of the protein suggesting involvement of the chaperone function of calreticulin (John *et al.*, 1998). Based on these observations it was proposed that the C-terminal tail of SERCA2b may be glycosylated *in vivo* and that calreticulin modulates SERCA2b Ca^{2+} transport activity by a direct interaction with the glycosylated C-terminal tail of the pump (John *et al.*, 1998). However, as of yet there is

no evidence demonstrating a direct interaction of SERCA2b with calreticulin. More recently, it was shown that calnexin, like calreticulin, can inhibit Ca^{2+} oscillations in a manner consistent with inhibition of SERCA2b function and that Asn1036 was also important for this inhibition (Roderick *et al.*, 2000). By immuno-precipitation it was demonstrated that calnexin interacts with the C-terminus of SERCA2b, an interaction that is drastically reduced when residue Asn1036 is mutated. Furthermore, Asn1036 does not appear to be glycosylated *in vivo* and it was proposed that a change in charge at the COOH terminus of the Ca^{2+} ATPase may be responsible for inhibiting this interaction. Phosphorylation of calnexin was also demonstrated to be important for interaction with SERCA2b. The following hypothesis was proposed - under resting conditions, calnexin is phosphorylated, allowing interaction with the C-terminal tail of SERCA2b, which keeps the pump in an inhibitory state. Upon mobilization of Ca^{2+} from internal stores, free $[Ca^{2+}]_C$ is increased resulting in the activation of a phosphatase that dephosphorylates calnexin. This would disrupt the interaction of calnexin with SERCA2b, allowing activation of SERCA2b and re-filling of the ER Ca^{2+} stores (Roderick *et al.*, 2000). Activation of the $InsP_3$ signaling pathway results in the activation of PKC, which is known to phosphorylate calreticulin (Rendon-Huerta *et al.*, 1999). Calreticulin associates with PKC under resting cellular conditions, while activation of PKC induces the phosphorylation of calreticulin. It would be interesting to determine if the interaction of calreticulin with SERCA2b is also regulated by phosphorylation.

- **Calreticulin and $InsP_3$-dependent Ca^{2+} release**

Once taken up by SERCA Ca^{2+} is released from the ER by the $InsP_3$/RyR receptors (Sorrentino and Volpe, 1993; Coronado *et al.*, 1994; Pozzan *et al.*, 1994; Furuichi and Mikoshiba, 1995; Sutko and Airey, 1996). Calreticulin deficient MEF cells have diminished agonist-mediated, $InsP_3$-dependent Ca^{2+} release from the ER (Mesaeli *et al.*, 1999) indicating that calreticulin may play a role in the functioning of the $InsP_3$ receptor pathway. Furthermore, down-regulation of calreticulin expression with antisense oligonucleotides results in the inhibition of bradykinin-mediated, $InsP_3$-dependent Ca^{2+} release from the ER in neuroblastoma X glioma NG-108-15 cells (Liu *et al.*, 1994). The domains of the $InsP_3$ receptor that are exposed to the ER lumen are glycosylated (Michikawa *et al.*, 1994). An attractive hypothesis is that calreticulin may bind to the glycosylated intraluminal loop(s) of the $InsP_3$ receptor and modulate Ca^{2+}-release (Michikawa *et al.*, 1994). Another possibility is that the protein may play an important role as chaperone of both $InsP_3$ receptor and/or molecules involved in the $InsP_3$ pathway. Results indicate the latter to be the case with calreticulin playing a role in the folding of the bradykinin receptor. Calreticulin-deficient cells show diminished Ca^{2+} release in response to bradykinin, yet release occurs upon direct activation with $InsP_3$ (Nakamura, 2001). Expression of the bradykinin receptor is not affected by changes in the level of calreticulin. However, impairment of bradykinin-induced Ca^{2+} release results from failure of bradykinin to bind it's cognate receptor, indicating that the bradykinin receptor may be incorrectly folded. In support of this, bradykinin binding and bradykinin-induced Ca^{2+} release are both restored by expression of full-length calreticulin in calreticulin deficient cells (Nakamura, 2001).

- **Calreticulin and Ca^{2+} store-operated Ca^{2+} release**

The depletion of intracellular Ca^{2+} stores activates a Ca^{2+} entry mechanism at the PM called capacitative Ca^{2+} entry (store-operated Ca^{2+} influx). The signal for activating the entry is unknown but likely involves either the generation or release, or both, from the ER of some diffusible signal. Store operated calcium influx is activated by the depletion of intracellular Ca^{2+} stores. Fasolato *et al* showed that calreticulin overexpression has no effect on store operated calcium influx in response to rapid, complete depletion of Ca^{2+} in the ER lumen (Fasolato *et al.*, 1998). They did, however, show that upon passive depletion of Ca^{2+} from the ER, overexpression of calreticulin delayed but did not inhibit store operated Ca^{2+} influx, and this delay was dependent on the C-domain of calreticulin (Fasolato *et al.*, 1998). More recently, Xu *et al* (Xu *et al.*, 2000) have shown that calreticulin can modulate store operated Ca^{2+} influx by controlling the extent of $InsP_3$-induced Ca^{2+} store depletion. Using deletion mutants they demonstrated that the C-domain of calreticulin is required to reduce the elevations of [free Ca^{2+}]$_C$ due to Ca^{2+} influx. This domain is also required for calreticulin to reduce the $InsP_3$-induced decline in the free Ca^{2+} in the ER lumen (Xu *et al.*, 2000). Therefore, by buffering the free Ca^{2+} in the ER lumen, calreticulin may prevent $InsP_3$ from depleting Ca^{2+} stores sufficiently to activate $InsP_3$–induced Ca^{2+} influx thus reducing Ca^{2+} influx (Xu *et al.*, 2000).

Other functional roles of calreticulin

Calreticulin modulates cell adhesion (Rojiani *et al.*, 1991; Dedhar *et al.*, 1994; Leung-Hagesteijn *et al.*, 1994; Coppolino *et al.*, 1995; Opas *et al.*, 1996a; Coppolino *et*

al., 1997; Fadel *et al.*, 1999), integrin-dependent Ca^{2+} signaling (Coppolino *et al.*, 1997). It also modulates steroid-sensitive gene expression both *in vitro* and *in vivo* (Burns *et al.*, 1994a; Burns *et al.*, 1994b; Dedhar, 1994; Dedhar *et al.*, 1994; St-Arnaud *et al.*, 1995; Wheeler *et al.*, 1995; Desai *et al.*, 1996; Michalak *et al.*, 1996; Burns *et al.*, 1997; Sela-Brown *et al.*, 1998). One major controversy in the calreticulin field concerns the mechanisms involved in calreticulin-dependent modulation of functions outside of the ER.

Calreticulin and cell adhesiveness.

Integrins are cell surface receptors that play a role in cellular adhesion. Upon ligand binding, integrins will cluster and form focal contacts (Schwartz *et al.*, 1991; Yamada and Miyamoto, 1995). These focal contacts are involved in the adhesion of cells to the extracellular matrix (Burridge and Chrzanowska-Wodnicka, 1996). The first evidence that calreticulin may be involved in integrin function and cell adhesion came from an *in vitro* experiment designed to identify cellular proteins that bind to the KxFF($^{K}/_{R}$)R synthetic peptide (Rojiani *et al.*, 1991), a region corresponding to the conserved amino acid sequence found in the C-terminal tail of the α-subunit of integrin (Williams *et al.*, 1994). These early experiments revealed that calreticulin binds, in a Ca^{2+}-dependent manner, to the KxFF($^{K}/_{R}$)R synthetic peptide affinity column (Rojiani *et al.*, 1991). Down-regulation of calreticulin expression results in a decrease in cellular adhesion and this was found to be integrin-mediated (Leung-Hagesteijn *et al.*, 1994). It was thus proposed that calreticulin may bind to the C-terminal, cytoplasmic tail of α-integrin and modulate its function (Dedhar, 1994; Leung-Hagesteijn *et al.*, 1994;

Coppolino *et al.*, 1995; Coppolino *et al.*, 1997; Coppolino and Dedhar, 1998). To regulate integrin activity *in vivo* by direct binding there must be focal contact-associated (cytoplasmic) calreticulin. Due to the lack of convincing evidence localizing calreticulin to the cytosol, researchers were forced to examine other potential means by which calreticulin could be affecting cell adhesion.

Recent reports indicate that calreticulin may influence cell adhesion indirectly, from the ER lumen, *via* modulation of gene expression of adhesion-related molecules (Opas *et al.*, 1996a; Fadel *et al.*, 1999) and/or by changes in the integrin-dependent Ca^{2+}-signaling (Coppolino *et al.*, 1997). For example, the level of calreticulin expression modulates cell adhesion by co-ordinating up-regulation of expression of vinculin and N-cadherin (Opas *et al.*, 1996a; Fadel *et al.*, 1999). Down-regulation of calreticulin causes inverse effects (Leung-Hagesteijn *et al.*, 1994; Opas *et al.*, 1996a). The changes in cell adhesion are also coincident with changes in the levels of protein tyrosine phosphorylation in cells differentially expressing calreticulin (Fadel *et al.*, 1999). The abundance of phosphotyrosine in cells over-expressing calreticulin is dramatically reduced in comparison to control cells. It is well documented that protein phosphorylation/dephosphorylation of tyrosine is a major mechanism for regulation of cell adhesion (Burridge and Chrzanowska-Wodnicka, 1996; Daniel and Reynolds, 1997; Hanks and Polte, 1997; Cox and Huttenlocher, 1998). Therefore, calreticulin may be able to modulate cellular adhesion through modulation of phosphotyrosine signaling pathways. Although the mechanism is still elusive, it is conceivable that the effects of calreticulin over-expression on cell adhesion may be due to calreticulin effects on a signaling pathway.

Calreticulin and steroid-sensitive gene expression.

Calreticulin binds to the DBD of steroid receptors and transcription factors containing the amino acid sequence KxFF($^{K}/_{R}$)R and prevents their interaction with DNA *in vitro* (Burns *et al.*, 1994a; Burns *et al.*, 1994b; Dedhar, 1994; Dedhar *et al.*, 1994; St-Arnaud *et al.*, 1995; Wheeler *et al.*, 1995; Winrow *et al.*, 1995; Desai *et al.*, 1996; Michalak *et al.*, 1996; Burns *et al.*, 1997; Sela-Brown *et al.*, 1998). With the exception of the PPAR/retinoid X heterodimers (Winrow *et al.*, 1995) transcriptional activation by glucocorticoid, androgen, retinoic acid and vitamin D_3 receptors *in vivo* is modulated in cells over-expressing calreticulin (Burns *et al.*, 1994b; Dedhar *et al.*, 1994; St-Arnaud *et al.*, 1995; Wheeler *et al.*, 1995; Desai *et al.*, 1996; Michalak *et al.*, 1996; Burns *et al.*, 1997; Sela-Brown *et al.*, 1998). It now appears that the DBD of the GR (amino acid sequence KxFF($^{K}/_{R}$)R) is functional as a novel NES (Holaska *et al.*, 2001). In binding this domain calreticulin may regulate the transcriptional activity of the GR through its export and may use this mechanism to regulate the transcriptional activity of other steroid hormone receptors (Holaska *et al.*, 2001).

ER luminal Ca^{2+} and calreticulin function

What are the consequences of Ca^{2+} release and reduction of the luminal $[Ca^{2+}]_{ER}$? Besides important effects on Ca^{2+} homeostasis, reduction of the $[Ca^{2+}]_{ER}$ (ER Ca^{2+} depletion conditions) leads to accumulation of misfolded proteins, increased expression of ER chaperones (Little *et al.*, 1994; Llewellyn *et al.*, 1995; Nguyen *et al.*, 1996; Waser *et al.*, 1997; Krause and Michalak, 1997) and ER-nucleus and ER-plasma membrane "signaling" (Little *et al.*, 1994; McMillan *et al.*, 1994; Llewellyn *et al.*, 1995; Nguyen *et*

al., 1996; Pahl and Baeuerle, 1997; Waser *et al.*, 1997; Chapman *et al.*, 1998; Sidrauski *et al.*, 1998; Kaufman, 1999). Ca^{2+} depletion inhibits ER-Golgi trafficking, blocks transport of molecules across the nuclear pore (Clapham, 1995; Greber and Gerace, 1995; Stehno-Bittel *et al.*, 1995; Macaulay and Forbes, 1996; Subramanian and Meyer, 1997) and affects chaperone function (Ivessa *et al.*, 1995; Srivastava *et al.*, 1995). Clearly, changes of the ER luminal $[Ca^{2+}]_{ER}$ have profound effects at multiple cellular sites including the structure and function of the ER luminal Ca^{2+} binding chaperones.

The dual function of the ER (Ca^{2+} storage and protein synthesis) is reflected in the functions of the individual proteins that reside there. One would hence expect that alterations in the $[Ca^{2+}]_{ER}$ would play an important role in the control of chaperone function of these proteins.

Hypothesis

The hypothesis upon which this work was based was that the structure and chaperone function of calreticulin would be modulated/controlled according to changes in the ER luminal environment. The major goal of my research was to investigate the interaction of calreticulin with other ER luminal chaperones and the role that ER luminal ions may play in these processes. Additionally, I sought to investigate the effect that changes in the ER luminal environment (specifically Ca^{2+}, Zn^{2+}, ATP and other ER luminal proteins) have on the conformation of calreticulin.

The specific research objectives of this thesis were to:-

- Analyze and identify interactions between calreticulin and other ER luminal chaperones
- Identify the domains of calreticulin important for these interactions
- Determine how the ER environment can affect these protein-protein interactions and what domains of calreticulin are responsible for these effects
- Determine the influence of the ER luminal environment on the conformation of calreticulin. Specifically look at the effects of Ca^{2+}, Zn^{2+} and ATP.
- Determine if calreticulin is an ATP-binding chaperone

The proposed work will provide important, new information on how the ER luminal environment can affect the function and structure of calreticulin. This information will lead to a greater understanding of how the dynamic ER luminal environment can affect many aspects of cellular function. Such findings will give us greater insight into how chaperones function and may lead to a greater understanding of the role of chaperones in disease states.

Chapter Two

Materials and Methods

A version of this chapter has previously been published: Corbett, E.F., Oikawa, K., Francois, P., Tessier, D.C., Kay, C., Bergeron, J.M., Thomas, D.Y., Krause, K-H., Michalak, M. (1999). Ca^{2+} regulation of interaction between endoplasmic reticulum chaperones. *J. Biol. Chem.* **274**: 10, 6203-6211. © *1999. The American Society for Biochemistry and molecular Biology.*

Corbett, E.F., Michalak, K.M., Oikawa, K., Johnson, S., Campbell, I.D., Eggleton, P., Kay, C., Michalak, M. (2000). Role of the endoplasmic reticulum luminal environment on the conformation of calreticulin. *J. Biol. Chem.* **275**: 35, 27177-27185. © 2000. *The American Society for Biochemistry and Molecular Biology.*

Materials

Reagents

The FluroTag FITC Conjugation Kit, MOPS, Pipes, DTPA, RNase, EGTA, ATP-Agarose, CL-2B Agarose, bovine IgG, Mg^{2+}-ATP, ADP, AMP, trypsin, V8 protease, cathepsin G, elastase were obtained from Sigma. DTT was purchased from ICN Biomedicals, Inc. Cascade Blue (CB) acetyl azide (cat. # C-2284) was from Molecular Probes, Inc. QuixSep microdialyzer was obtained from Membrane Filtration Products, Inc. Sephadex G-25M was from Pharmacia. SDS-PAGE reagents and molecular weight markers were from Bio-Rad. The ECL detection kit for Western blotting was from Amersham. All chemicals were of the highest grade available.

Antibodies

Goat anti-calreticulin antibodies were extensively characterized (Milner *et al.*, 1991). This antibody recognizes the C-domain but not the N-domain of calreticulin. Antibodies against the N-terminal synthetic peptide of calreticulin were a generous gift of Dr. K-H. Krause, University of Geneva. This antibody recognizes the first 12 amino acids of mature calreticulin and has also been extensively characterized (Van Delden *et al.*, 1992; Stendahl *et al.*, 1994; Mery *et al.*, 1996). Horse radish peroxidase-conjugated rabbit anti-goat and goat anti-rabbit IgGs were from Jackson ImmunoResearch Laboratories.

Antibody binding was detected with appropriate peroxidase-conjugated secondary antibodies diluted 1:10,000, followed by an ECL reaction and exposure to X-ray film. Images of the Coomassie blue stained gels and X-ray films were scanned and processed for publication using Adobe Photoshop v. 5.5 software.

Expression and Purification of Recombinant ERp57

Human ERp57 was a generous gift from Dr. David Thomas (McGill University, Montreal, Quebec). It was expressed in *E. coli* and purified as described previously (Zapun *et al.*, 1998).

Methods

Purification of PDI

PDI was isolated from bovine liver by a procedure of Lambert and Freedman (Lambert and Freedman, 1983) and modified as described by Baksh *et al* (Baksh *et al.*, 1995a). Briefly, 500 g of

frozen bovine liver was thawed overnight at 4°C and homogenized at full speed for 4 *x* 30 seconds in 1 L of buffer containing 100 mM sodium phosphate, pH 7.5, 5 mM EDTA, 1% v/v Triton X-100, 0.5 ml SL protease inhibitors/100 ml buffer, 0.5 mM PMSF and 0.5 mM benzamidine. The homogenate was strained, centrifuged at 18,000g for 30 minutes, and the pellet discarded. The supernatant was placed in a water bath at 70°C with constant stirring and the temperature of the extract was allowed to reach 54°C ± 1 °C, where it was maintained for 15 minutes. Afterwards it was cooled on ice and centrifuged at 18,000g for 40 minutes as before. The pellet was discarded and 351g/L of solid ammonium sulfate was added to reach 55% saturation of ammonium sulfate. After stirring for 30 minutes, the precipitated supernatant was centrifuged at 18,000g for 30 minutes. The pellet was discarded and 218g/L of solid ammonium sulfate was added to reach 85% saturation, stirred at 4°C for 30 minutes, and then centrifuged as before. The supernatant was discarded and the pellet was re-suspended in 20 mls of 25 mM sodium

citrate, pH 5.3 and dialyzed overnight against the same buffer. This was followed by centrifugation at 12,000g for 12 minutes. Further purification of PDI was conducted on a carboxymethylcellulose (CM) cation exchange column. The sample was loaded onto the CM column, previously equilibrated with 25 mM citrate, pH 5.3 and then eluted with the same buffer at a flow rate of 3-4 ml/minute. Eluted proteins (containing PDI and other acidic proteins) were collected and analyzed on SDS-PAGE, followed by immuno-blotting with anti-PDI antibodies. Peaks containing PDI were pooled, concentrated, and solid ammonium sulfate was added to achieve 100% saturation. The solution was stirred at 4°C for 30 minutes, and then centrifuged at 18,000g for 40 minutes. The precipitated sample was then centrifuged at 18,000g for 30 minutes, the supernatant discarded and the pellet re-suspended in 15 ml of 20 mM sodium phosphate, pH 6.3 and dialyzed extensively. This was followed by centrifugation at 12,000g for 12 minutes. The sample was then applied to a Mono Q cation FPLC column, previously equilibrated with 20 mM sodium phosphate, pH 6.3. Proteins were eluted in a linear salt gradient from 0-700 mM NaCl in the same buffer and 2.5 ml fractions were collected. Fractions containing PDI were confirmed by its mobility on SDS-PAGE and its immuno-reaction with antibodies against PDI.

Expression and purification of GST fusion proteins

The expression and purification of GST fusion is briefly described here. The recombinant domains of calreticulin (N-, P- and C-domains) and recombinant GST were previously cloned into the pGEX-3X expression vector to form pGEX-N, pGEX-P and pGEX-C. These constructs were then transformed and expressed in the protease-

deficient *E. coli* strain, BNN103. The resulting fusion proteins had the GST protein fused to its amino terminus (Baksh and Michalak, 1991; Baksh *et al.*, 1992). The following expression and purification protocol applies to the production of GST alone or GST-fusion proteins. Transformed BNN103 cells were grown in LB broth containing 100 μg/ml of ampicillin to mid-log phase (A_{600}= 0.6-1.0). To induce production of the fusion protein, 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to the growing culture. The cells were grown for an additional 4 hours, after which they were harvested by centrifugation at 3,500g for 15 minutes at 4°C. The resulting pellet was re-suspended in 1.0 % Triton X-100 in PBS and lysed using a French Press set at 1,000 p.s.i. Lysates were then centrifuged for 10 minutes at 10,000g to remove cellular debris and insoluble matter. The supernatants were applied to a 40 ml glutathione-sepharose 4B column (2 cm x 12 cm; flow rate 1 ml/minute; capacity 7.5 mg/ml of beads) previously equilibrated with 0.1 % Triton X-100 in PBS. The column was washed with 5 column volumes of Triton X-100/PBS buffer. Bound fusion proteins were eluted with 5 mM glutathione in 50 mM Tris-HCl, pH 8.0. Approximately 2.5 ml fractions were collected, analyzed by SDS-PAGE and Western blotting.

Purification of canine pancreatic calreticulin

Canine pancreatic calreticulin was purified by ammonium sulfate precipitation procedures as previously described and is briefly described here (Milner *et al.*, 1991; Baksh *et al.*, 1992;). Canine pancreases were homogenized in 1 ml/g tissue of buffer containing 100 mM KH_2PO_4, pH 7.1, 1 mM EDTA and SL inhibitors, with 2.66 M $(NH_4)_2SO_4$ (55% saturation) in a blender for 1 minute. The homogenate was then

centrifuged at 12,000g for 30 minutes. The supernatant was filtered through gauze and saved. The pellet was re-suspended in half the original volume of the same buffer and re-homogenized. The homogenate was centrifuged again as before. The resulting supernatant was filtered and combined with the first supernatant. The pellet at this point was discarded. 200 g of $(NH_4)_2SO_4$ was added per liter of supernatant with stirring to reach 85% saturation. This was left to stir for 30 minutes. The pH was adjusted to 4.4 by adding 85% phosphoric acid drop-wise. The resulting suspension was stirred for 4 hours at 4°C and then centrifuged at 12,000g for 30 minutes using the same rotor as before. The supernatant was discarded and the pellet was dissolved in 100 mM KH_2PO_4, pH 7.1, 1 mM EGTA. This was then dialyzed twice for 24 hours each time against 100 mM KH_2PO_4, pH 7.1, 1 mM EGTA and 50 mM NaCl. The suspension was centrifuged at 12,000g for 30 minutes as before. The supernatant was applied directly to a 60 ml DEAE-Sephadex A-50 column (3 cm x 12 cm; flow rate ~ 0.6 ml/min; capacity 200 mg) previously equilibrated with 100 mM KH_2PO_4, pH 7.1, 1 mM EGTA and 50 mM NaCl. The column was washed with 5 column volumes of the same buffer. A linear gradient of 50-750 mM NaCl (0.83 mM NaCl/minute) in the phosphate buffer was applied to the column and 7 ml fractions collected. Fractions were analyzed by SDS-PAGE and fractions containing calreticulin were pooled and dialyzed for 16 hours against 10 mM KH_2PO_4, pH 7.0. The dialyzed calreticulin was loaded onto a 50 ml hydroxylapatite column (2 cm x 15 cm; flow rate ~ 0.5 ml/minute; capacity 20 mg) and further purified. The column was washed with three column volumes of 10 mM KH_2PO_4, pH 7.0 and the remaining calreticulin bound to the column was eluted in a linear gradient of 10-1000 mM KH_2PO_4, pH 7.0 (~1.25 mM KH_2PO_4/minute). Fractions were collected and

analyzed by SDS-PAGE. Pure fractions were pooled, concentrated using an Amicon concentrator with a YM-30 membrane and verified by western blotting with anti-calreticulin antibodies.

Expression and purification of recombinant calreticulin in *Pichia* protein expression system

Recombinant calreticulin was expressed and purified according to Andrin *et al.* (Andrin *et al.*, 2000) and is briefly described here. The cDNA of full-length rabbit calreticulin was cloned into pPIC-9 and transformed into the *Pichia* KM71 strain as recommended by the manufacturer. Transformants were grown in buffered minimal glycerol media for 18 hours at 30°C. Cells were then harvested by centrifugation at 3,000g for 5 minutes at room temperature. To induce protein expression, cells were suspended in a buffered minimal medium containing 0.5% methanol. Protein expression and secretion were monitored at 24, 48 and 72 hours. A 0.5% concentration of methanol was maintained by addition of 1 ml of 100% methanol every 24 hours. Optimum expression of calreticulin was observed after 72 hours of methanol induction. To purify the recombinant calreticulin, the medium obtained after 72 hours of methanol induction was concentrated eightfold using an Amicon apparatus with a filter of 30,000 molecular weight cutoff. Concentrated protein sample was loaded onto a DEAE-Sepharose column equilibrated with a solution of low-salt buffer containing 50 mM NaCl, pH 7.1, 100 mM KH_2PO_4, 1 mM EDTA. The recombinant protein was eluted with a salt gradient from 50-800 mM NaCl in a buffer containing 100 mM KH_2PO_4, 1 mM EDTA. The fractions containing recombinant calreticulin were pooled and concentrated using Amicon

apparatus and a filter of 30,000 molecular weight cutoff. Several washes of the Amicon concentrator were carried out to exchange the buffer of the protein sample to 20 mM NaH_2PO_4, Na_2HPO_4, pH 7.0. The concentrated protein fraction was then further purified using a Resource Q FPLC column (Pharmacia). Approximately 100 mg of protein was loaded onto the column and washed with three column volumes of a buffer containing 20 mM NaH_2PO_4, Na_2HPO_4, pH, 7.0. The protein was eluted using a salt gradient from 0-780 mM NaCl in the same buffer. One ml fractions were collected and analyzed by SDS-PAGE. The fractions containing recombinant calreticulin were pooled and concentrated using the Amicon apparatus. Identity of the recombinant protein was verified by NH_2-terminal amino acid analysis and by its reactivity with anti-calreticulin antibodies as determined by Western blot analysis. The NH_2-terminal amino acid sequence of full-length calreticulin expressed in *Pichia* was NH_2-E-A-Y-V-E-F-^{1}E-P-V-V-Y-F-L-, where the underlined residues correspond to the amino acid sequence of mature calreticulin. Biochemical and biophysical analysis of the recombinant calreticulin using mass spectroscopy, CD analysis, disulfide mapping, Ca^{2+} binding measurements and antibody reactivity revealed that it was identical to the native, tissue purified protein (Andrin *et al.*, 2000).

Fluorescence labeling of proteins with Cascade Blue

Proteins were labeled with Cascade Blue (CB) acetyl azide by adaptation to a FluroTag FITC Conjugation Kit procedure as recommended by the manufacturer. Briefly, CB (1.11 mg/ml) was dissolved in 100 mM sodium carbonate-bicarbonate buffer pH 9.0. Six hundred μg of purified PDI or ERp57 (2.6 mg/ml) in a 100 mM sodium

carbonate-bicarbonate buffer, pH 9.0 was used directly for labeling. The dye was added drop-wise to the protein mixture with constant stirring. The reaction vial was incubated in the dark for two hours at room temperature with gentle stirring. Labeled proteins were separated from the free dye on a Sephadex G-25M column (3.5 ml, bed height 2.6 cm) previously equilibrated with PBS. The reaction mixture was applied and fractions (0.25 ml each) were eluted with PBS. The fluorescence of each fraction was determined at the excitation wavelength 385 nm and the emission wavelength 430 nm using a Perkin Elmer spectrophotometer. Fractions containing labeled protein were combined and used directly for protein-protein interaction studies. Determination of the stoichiometry of the labeling revealed that there were 4 and 5 molecules of CB conjugated per each molecule of ERp57 and PDI, respectively, indicating that over 90% of the protein was labeled with the dye.

Fluorescence measurements

Fluorescence measurements were performed at an excitation wavelength 385 nm, (slit, 15 nm) and an emission wavelength 430 nm, (slit, 15 nm) at room temperature using a Luminescence Spectrometer LS50B (Perkin Elmer). Fluorescence intensities were measured with constant stirring in 1.5 ml of a binding buffer containing 10 mM Mops, pH 7.0, 100 mM KCl, 2 mM $MgCl_2$, 0.5 mM EGTA. Appropriate proteins and/or ions were added to the reaction mixture and the corresponding changes in fluorescence intensity were monitored. Quantum yields of CB or CB labeled proteins were calculated from the emission spectra (420-460 nm) obtained at the $\lambda_{excitation}$ of 385 nm. Initial rates

of the protein-protein interaction and time constants were calculated using an exponential decay function using Origin v. 4.1 software.

Circular dichroism measurements

For circular dichroism (CD) analysis proteins were dialyzed for 16 hours against a buffer containing of 25 mM Pipes, pH 6.8, 100 mM NaCl, 1 mM EGTA, 1 mM DTT. Dialysis was performed using Spectra/Por dialysis tubing (cutoff 12-14 kDa) in a QuixSep Microdialyzer. CD measurements were carried out on a Jasco J-720 spectropolarimeter (Jasco Inc, Easton, MD), interfaced to an Epson Equity 386/25 and controlled by Jasco software. The thermostabled cell holder was maintained at 25°C with a Lauda RMS circulatory water bath (Lauda, Westbury, N.Y.). The instrument was routinely calibrated with ammonium d-(+)-10 camphor sulfonate at 290.5 and 192 nm. Each sample was scanned ten times and noise reduction applied to remove the high frequency before calculating molar ellipticities. The voltage to multiplier was kept below 500 V to prevent distortion of the CD spectrum. The cell pathlength used was 0.02 cm and the protein concentrations were 0.6 mg/ml in the far ultraviolet. Concentrations were determined on a Cary 3 UV-visable spectrophotometer and were corrected for light scattering. Molar extinction coefficients were calculated from tyrosine and tryptophan compositional values and were 43780, 45040 and 81480 for ERp57, PDI and calreticulin, respectively. Molar ellipticities were calculated from the equation:

$$[\theta] = \theta_{obs}/10 \times l \times c,$$

where: θ_{obs} is in millidegrees, l is the pathlength in cm and c is the concentration in moles/liter x number of amino acids in the sequence. The unit for molar ellipticity is degree-centimeter squared per decimole. The CD spectra were analyzed for secondary

structure elements by the Contin ridge regression analysis program of Provencher and Glöckner (Provencher and Glöckner, 1981)

Sedimentation velocity measurements

Sedimentation velocity experiments were carried out at 20°C and 50,000 rpm using XLI Analytical Ultracentrifuge and Interference optic following the procedures outlined in the instruction manual published by Spinco Business Center of Beckman Instruments, Inc. Palo Alto, Ca (1997). Four hundred µl of sample solution and 400 µl of dialysate were loaded into 2 sector CFE centerpiece sample cells containing sapphire windows. Runs were performed for 4 hours during, which a minimum of 30 scans were taken. The sedimentation velocity data was analyzed using the Second Moment Method contained in the Beckman Analysis Program (Optima XL-A/XL-I Data Analysis Software, version 4.0; Spinco Business Center of Beckman Instruments, Inc. Palo Alto, Ca.) to determine the sedimentation coefficient from the following relationship:

$$S\varpi^2 = d\ln r/dt$$

where: S = observed sedimentation coefficient; ϖ = angular velocity; r = radial position of the solvent/protein boundary; and t = the time in seconds. By measuring the movement of the boundary over time and plotting ln r versus $\varpi^2 t$, a straight line should be obtained with the slope = S.

The program Sednterp (Sedimentation Interpretation Program, version 1.01, created by David B. Haynes, Magdelan College, Tom Laue, University of New Hampshire, and John Philo, Amgen, Copyright © 1995-1998) was then used to calculate $S_{20,w}$, according to the following relationship:

$$S_{20,w} = s\,(\eta_t/\eta_{20})\,(\eta_{sol}/\eta_w)\,(1 - \upsilon\rho_{20,\,w} / 1 - \upsilon\rho_{t,\,sol})$$

where: $S_{20,w}$ = sedimentation coefficient corrected to water 20°C; s = observed sedimentation coefficient; η_t = viscosity of water at run temperature; η_{20} = viscosity of water at 20°C; η_{sol} = viscosity of sample solution at known temperature t'; η_w = viscosity of water at t' degrees; $\rho_{20,w}$ = density of water at 20°C; $\rho_{t,sol}$ = density of sample solution at run temperature; and υ is the partial volume of the solute.

Proteolytic digestions

Purified, recombinant calreticulin expressed in *Pichia,* was incubated with trypsin at 1:100 (trypsin/protein; w/w) at 37°C and in the presence or absence of varying concentrations of Ca^{2+}, Zn^{2+}, Mg-ATP (Chapter Four - as indicated in the legends to the Figures). Tryptic digestion of calreticulin was also carried out in the presence of the purified PDI (50 μg) and ERp57 (75 μg). Aliquots were taken at indicated time points and the reaction was stopped by addition of the Laemmli sample buffer (Laemmli, 1970). Calreticulin (15 μg) was also digested in the absence (5 mM EDTA) or in the presence of Ca^{2+} (2 mM Ca^{2+}) with elastase, V8 protease, and cathepsin G at calreticulin:enzyme ration of 20:1 or 320:1 (w/w) for 2 hours at 37°C at pH 6.4 (elastase) or pH 7.4 (V8 protease, and cathepsin G). The proteins were separated by 10% or 15% SDS-PAGE (Laemmli, 1970), and either stained with Coomassie blue or transferred onto a nitrocellulose membrane (Towbin *et al.*, 1979) and probed with the indicated anti-calreticulin antibodies or antibodies to PDI. Standards were Bio-Rad low range molecular weight proteins.

ATP-agarose chromatography

ATP affinity chromatography was carried out using ATP-Agarose and CL-2B Agarose as the control. Calreticulin (5 μg) was incubated at 37°C for 60 minutes with ATP-Agarose or CL-2B agarose beads (0.7 g/ml) in a binding buffer containing 10 mM Mops, pH 7.0, 100 mM KCl, 2 mM $MgCl_2$, 0.5 mM EGTA, and 2 mg of bovine IgG (to block non-specific binding). Beads were then placed in a glass column followed by extensive washing with a binding buffer to remove unbound proteins. ATP bound proteins were eluted with a binding buffer containing 5 mM Mg^{2+}-ATP. Proteins associated non-specifically with the ATP-agarose were subsequently eluted with 2 M NaCl. Eluted proteins were precipitated with acetone at –20°C for 16 hours, separated on 15% SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were probed with anti-calreticulin antibodies as described above.

SDS-Polyacrylamide gel electrophoresis and Western Blotting

SDS-PAGE was performed according to Laemmli (Laemmli, 1970). 10% and 15% gels were used. The low molecular weight protein markers (BioRad) used were; phorphorylase (97,400), bovine serum albumin (66,200). Ovalbumin (42,700), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,400). After gel electrophoresis, for detection of separated proteins the gels were either stained with Coomassie blue or electrophoretically transferred to nitrocellulose membrane using a semi-dry transfer apparatus (Towbin *et al.*, 1979).

After transfer, nitrocellulose membranes were stained with Ponceau S to verify that the protein transfer had occurred. The membranes were blocked in 5% milk powder

dissolved in PBS for 1 hour at room temperature with shaking. The blocked membranes were then incubated with primary antibody in 1% milk powder in PBS for 1 to 2 hours. Primary antibodies used were diluted as follows: goat anti-calreticulin (serum), 1:300; rabbit anti-calreticulin, 1:500. After incubation with the primary antibody the membranes were washed twice in 0.05% tween-20 in PBS followed by one 10 minute wash in PBS alone. The membranes were then incubated with the appropriate horseradish peroxidase conjugated secondary antibody diluted 1:10,000 in 1% milk powder in PBS for 1 hour. The wash steps were then repeated. Detection of the bound antibodies was by the chemiluminescent ECL detection system (Amersham).

Determination of protein concentration

Protein was determined by the method Bradford (Bradford, 1976). Bovine serum albumin was used as a standard in these assays.

Protein sequencing

NH_2-terminal sequence analysis of native and recombinant proteins was carried out on samples, which were electroblotted to Immobilon (polyvinylidene difluoride) membranes (Matsudaira, 1987). Protein sequencing was carried out in the Department of Biochemistry, University of Victoria, British Columbia.

Refolding of RNase B

Refolding of RNase B in the presence of CB labeled proteins was carried out as described previously (Zapun *et al.*, 1998), and will be briefly described here. After monoglucosylation, refolding was initiated by dissolving freeze-dried reduced RNase B

(~60 μM) in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM $CaCl_2$, 0.5mM oxidized glutathione. This was in the presence or absence of ~ 12 μM of ΔTMC (recombinant truncated calnexin corresponding to residues 21-487 after signal peptide cleavage) or calreticulin, and ~ 4 μM of either ERp57 or PDI at 25°C. Aliquots were withdrawn at various time points, reacted for 5 minutes at room temperature with 0.25 volumes of 0.5 M iodoacetamide in 1.5 M Tris-HCl (pH 8.7), and then stored on ice prior to analysis by low pH non-denaturing PAGE.

ATP hydrolysis

ATP hydrolysis was carried out using a coupled enzyme system as described previously (Poch *et al.*, 1998).

Miscellaneous

All recombinant techniques were conducted according to standard protocols (Ausabel, 1989). Free Ca^{2+} concentrations were calculated using Max Chelator, Winmaxc v. 1.70.

Chapter Three

Calcium Regulation of Interactions between Endoplasmic Reticulum Luminal chaperones

A version of this chapter has previously been published: Corbett, E.F., Oikawa, K., Francois, P., Tessier, D.C., Kay, C., Bergeron, J.M., Thomas, D.Y., Krause, K-H., Michalak, M. (1999). Ca^{2+} regulation of interaction between endoplasmic reticulum chaperones. *J. Biol. Chem.* **274**: 10, 6203-6211. © *1999. The American Society for Biochemistry and Molecular Biology.*

Introduction

Earlier studies from our laboratory have identified calreticulin as one of the major Ca^{2+} binding, multifunctional proteins of the ER membrane and documented that the protein interacts with PDI (Baksh *et al.*, 1995a; Michalak, 1995). PDI is an abundant, ER luminal protein which catalyzes a variety of thiol/disulfide exchange reactions (Noiva and Lennarz, 1992; Freedman *et al.*, 1994). The disulfide isomerase activity of PDI is decreased in the presence of either calreticulin or calnexin (Baksh *et al.*, 1995a; Zapun *et al.*, 1998). Another ER chaperone, ERp57, a homologue of PDI, displays thiol-dependent reductase (Hirano *et al.*, 1995), and cysteine-dependent protease activities (Urade *et al.*, 1997). Results from High's group reported that ERp57 can be cross-linked to monoglucosylated glycoproteins that are substrates for calnexin and calreticulin (Elliott *et al.*, 1997; Oliver *et al.*, 1997) suggesting that ERp57 may also be a lectin-like chaperone. Zapun *et al* (Zapun *et al.*, 1998) identified functional complexes between ERp57, calreticulin and calnexin and showed that disulfide isomerase activity of ERp57 is much greater in the presence of calreticulin or calnexin suggesting a functional association between these proteins. However, direct interaction between calreticulin and ERp57 has not yet been reported.

In addition to their chaperone function, the majority of ER resident proteins, including calreticulin and PDI, bind Ca^{2+} and Zn^{2+} and contribute to the Ca^{2+} storage capacity of the ER and cellular Ca^{2+} homeostasis (Khanna *et al.*, 1986; Milner *et al.*, 1992; Racchetti *et al.*, 1994; Baksh *et al.*, 1995a; Baksh *et al.*, 1995b; Baksh *et al.*, 1995c; Bastianutto *et al.*, 1995; Mery *et al.*, 1996; Krause and Michalak, 1997; Lievremont *et al.*, 1997; John *et al.*, 1998; Lucero *et al.*, 1998; Meldolesi and Pozzan,

1998a). Therefore, changes in the free $[Ca^{2+}]_{ER}$ due to Ca^{2+} release *via* $InsP_3$ and RyR receptors and Ca^{2+} uptake *via* Ca^{2+}-ATPAse (SERCA) (Pozzan *et al.*, 1994) are expected to play an important role in the control of chaperoning and other functions of these proteins (Krause and Michalak, 1997; Meldolesi and Pozzan, 1998a). How chaperones interact to facilitate protein folding and what is a role of the ER luminal ions in these processes is not known.

The goal of this study was to examine interactions between calreticulin and two related ER membrane chaperones: PDI and ERp57. We set out to investigate the role that each of calreticulins domains plays in these interactions and the effect that free $[Ca^{2+}]_{ER}$ would have on these protein-protein interactions.

Results

Changes in fluorescent intensity of Cascade Blue (CB) are indicative of conformational changes in PDI and ERp57

CB acetyl azide reacts with aliphatic amines in proteins to yield stable carboxamides, is highly fluorescent and resists quenching upon protein conjugation (Whitaker *et al.*, 1991). One unique feature of CB that we discovered is that it has different fluorescence intensity depending on the polarity of the solvent used (Fig. 3-1 and Table 3-1). For example, CB alone had relatively high fluorescence intensity in PBS and water but significantly lower fluorescence in non-polar solvents such as *n*-propanol and *n*-butanol (Fig. 3-1 and Table 3-1). This observation suggested to us that the dye might also be sensitive to exposure to the hydrophilic or hydrophobic environments in a protein. To test this ER luminal chaperones, PDI and ERp57, were labeled with CB to generate CB-PDI and CB-ERp57, respectively. Labeled proteins displayed emission and

Figure 3-1: Emission spectra of CB in different solvents at $\lambda_{excitation}$ of 380 nm

Emission spectra analysis of CB was carried out in PBS, methanol, *n*-propyl alcohol (*n*-propanol) and *n*-butyl alcohol (*n*-butanol) at the excitation wavelength of 380 nm. Data provided by Patrice Francois.

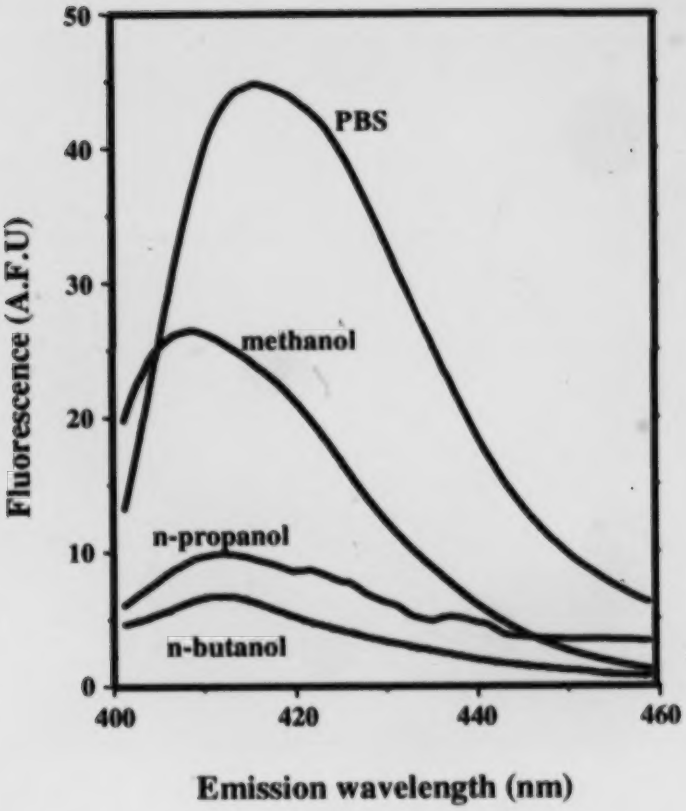

50
40
30
20
10
0
Fluorescence (A.F.U)
PBS
methanol
n-propanol
n-butanol
400
420
440
460
Emission wavelength (nm)

Table 3-1

Relative quantum yield of Cascade Blue acetyl azide and CB coupled to ERp57 (CB-ERp57) or PDI (CB-PDI) in different solvents of various polarity

Solvent	CB		CB-ERp57		CB-PDI	
	Q	λ_{max} *nm*	Q	λ_{max} *nm*	Q	λ_{max} *nm*
PBS	1	416	1	436	1	435
Water	0.94	416	0.83	436	1.37	435
Ethanol	0.68	408	0.63	431	1.13	430
Methanol	0.62	407	0.16	435	0.87	430
25% glycerol	0.38	415	0.92	437	0.17	441
50% glycerol	0.28	415	0.19	438	0.38	437
Propyl alcohol	0.005	412	0.34	433	0.61	432
Butanol	0.0037	413	0.22	438	0.29	433

excitation spectrum similar to the un-conjugated flurophore. Similar to CB alone, fluorescence intensity of the protein-conjugated CB (CB-ERp57 and CB-PDI) had different relative quantum yields in various solvents (Table 3-1) suggesting that the protein-conjugated dye had a different fluorescence intensity depending on whether it was exposed to a polar or a non-polar microenvironment.

Native PDI and ERp57 catalyze a variety of thiol/disulfide exchange reactions (Noiva and Lennarz, 1992; Freedman *et al.*, 1994; Hirano *et al.*, 1995; Zapun *et al.*, 1998) and their chaperone activity can be estimated by their ability to refold RNase B (Zapun *et al.*, 1998). Furthermore, ERp57 disulfide isomerase activity is increased in the presence of calreticulin or calnexin, while PDI's is inhibited (Zapun *et al.*, 1998). It was, therefore, important to demonstrate that CB labeling of PDI and ERp57 does not interfere with the function of these chaperones. We examined the disulfide isomerase activity of CB-PDI and CB-ERp57 on the refolding of RNase B in the presence and absence of calreticulin and calnexin. Figure 3-2 shows that similar to native PDI and ERp57 (Zapun *et al.*, 1998), CB-PDI and CB-ERp57 catalyzed refolding of RNase B indicating that labeled proteins retained their chaperone activity. Refolding of RNase B catalyzed by CB-PDI or CB-ERp57 was not influenced by changes of Ca^{2+} or Zn^{2+} concentration.

Next we tested effects of ions (Ca^{2+} and Zn^{2+}) and purified calreticulin on CB alone and on CB labeled proteins. Fluorescence intensity of CB alone did not change in the presence of Ca^{2+}, Zn^{2+} or purified calreticulin (Fig. 3-3A). However, the amplitude of fluorescence of the labeled proteins was sensitive to ion-induced conformational changes. Figures 3-3B and 3-3C show that addition of Zn^{2+} to CB-PDI or CB-ERp57 resulted in significant decrease in fluorescence intensity of the labeled proteins. To test whether

Figure 3-2: Refolding of RNase B catalyzed by CB-PDI and CB-ERp57

Refolding of reduced RNase B was carried out in the presence of CB-ERp57 (ERp57), CB-PDI (PDI) or without the catalyst (CTL) and in the presence of calnexin (CNX) or calreticulin (CRT). The reaction was terminated after indicated times and the RNase B conformation was examined by non-denaturing polyacrylamide gel electrophoresis. Unfolded RNase B has the slowest mobility (U), and the native form (N) has the greatest. Data provided by Daniel Tessier.

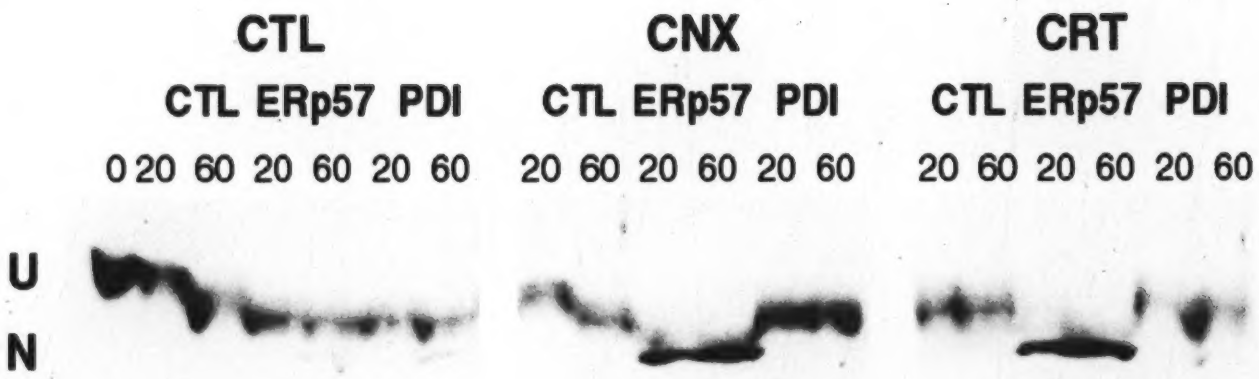
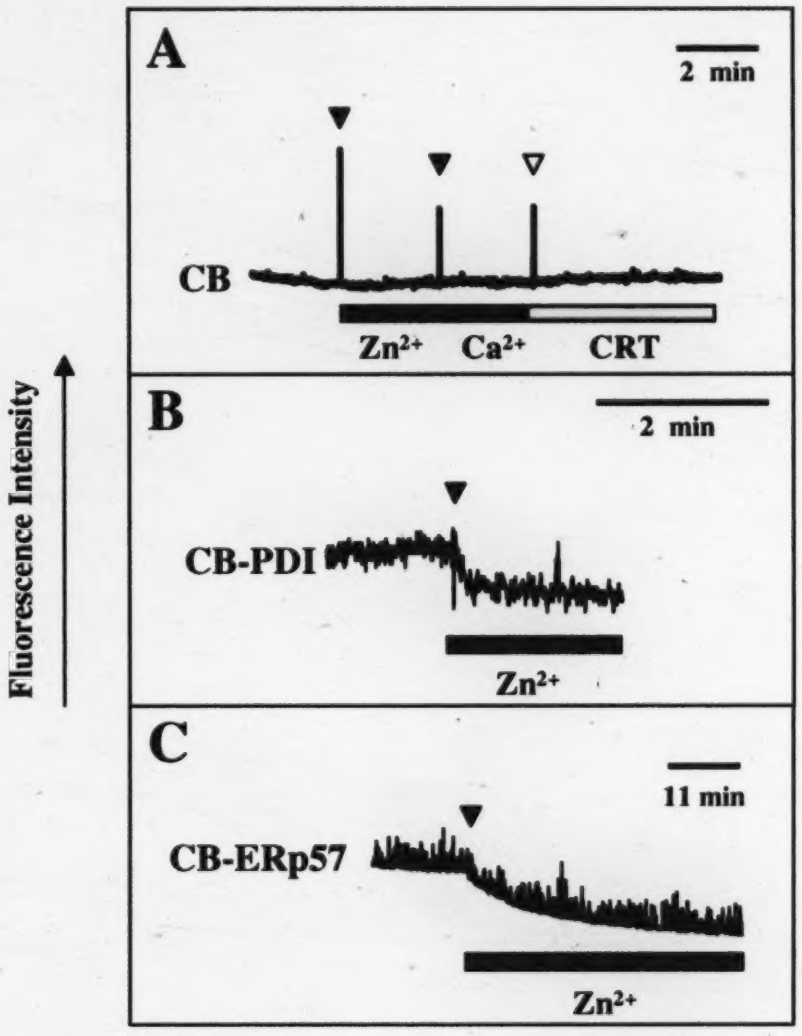

CTL
CNX
CRT
CTL ERp57 PDI
CTL ERp57 PDI
CTL ERp57 PDI
0 20 60 20 60 20 60
20 60 20 60 20 60
20 60 20 60 20 60
U
N

Figure 3-3: Zn^{2+}-induced conformational changes in Cascade Blue labeled PDI and ERp57

CB labeling of PDI and ERp57 was carried out as described under "Methods", Chapter Two. Changes in the fluorescence intensities of CB (A) and CB-PDI (B) and ERp57 (C) were monitored in the absence or presence of 500 μM Zn^{2+} (A & B), and 1 mM Zn^{2+} (C). In (A), the effects of Zn^{2+}, 1 mM Ca^{2+} and purified pancreatic calreticulin on fluorescence intensity of CB alone was examined. In (B & C); effects of 500 μM Zn^{2+} on CB-PDI and CB-ERp57. Bars represent the duration of incubation with ions or purified proteins. Arrowheads depict the time of addition.


A
2 min
CB
Zn2+
Ca2+
CRT
B
2 min
CB-PDI
Zn2+
C
11 min
CB-ERp57
Zn2+
Fluorescence Intensity

changes in fluorescence intensity induced by Zn^{2+} (Fig. 3-3B and 3-3C) reflected conformational alterations in CB-PDI and CB-ERp57 we carried out CD analysis of the purified proteins. Figure 3-4 reveals that the CD spectra of ERp57 and PDI are very similar in shape, having minima at 219 and 210 nm with molar ellipticity values of –10330° and –10180° at 219 nm and -9490° and -9670° at 210 nm, respectively. The Contin version program for calculating secondary structural elements (Table 3-2) indicates the α-helical content for both proteins was 25-30% while the combined β-sheet and β-turn was ~ 50%. The values for PDI are similar to those reported in previous studies by Wetterau *et al* (Wetterau *et al.*, 1991a; Wetterau *et al.*, 1991b). Both proteins underwent a conformational change upon addition of Zn^{2+} with a loss in the amount of α-helix and a concomitant increase in β-sheet-β-turn. Similarly, pancreatic calreticulin showed no change upon addition of Ca^{2+} but underwent a reduction in α-helix and an increase in combined β-sheet-β-turn upon addition of 2 mM Zn^{2+}, a finding also reported earlier by Khanna *et al* (Khanna *et al.*, 1986). The apo-protein has ~ 10% α-helix and 50% β-sheet-β-turn, upon analysis (Table 3-2). We concluded that Zn^{2+}-dependent changes in the fluorescence intensity of CB-ERp57 and CB-PDI (Fig. 3-3) may be due to conformational changes in the proteins (Fig. 3-4) resulting from exposure of the conjugated CB to protein microenvironments of a different polarity (Figure 3-1).

Calcium regulates interactions between calreticulin and PDI

Can the observed conformational changes in the CB-PDI and CB-ERp57 be applied to study protein-protein interactions? We first investigated the interaction between CB-PDI and calreticulin (Fig. 3-5). CB-PDI was incubated with Zn^{2+} to induce

Figure 3-4: CD analysis of ER lumenal proteins

CD spectra of purified calreticulin, PDI and recombinant ERp57 were carried out as described under "Methods", Chapter Two. The data are plotted as molar ellipiticity versus wavelength for the proteins in the absence (——) and presence of Zn^{2+} (-----). Kim Oikawa performed the CD analysis.

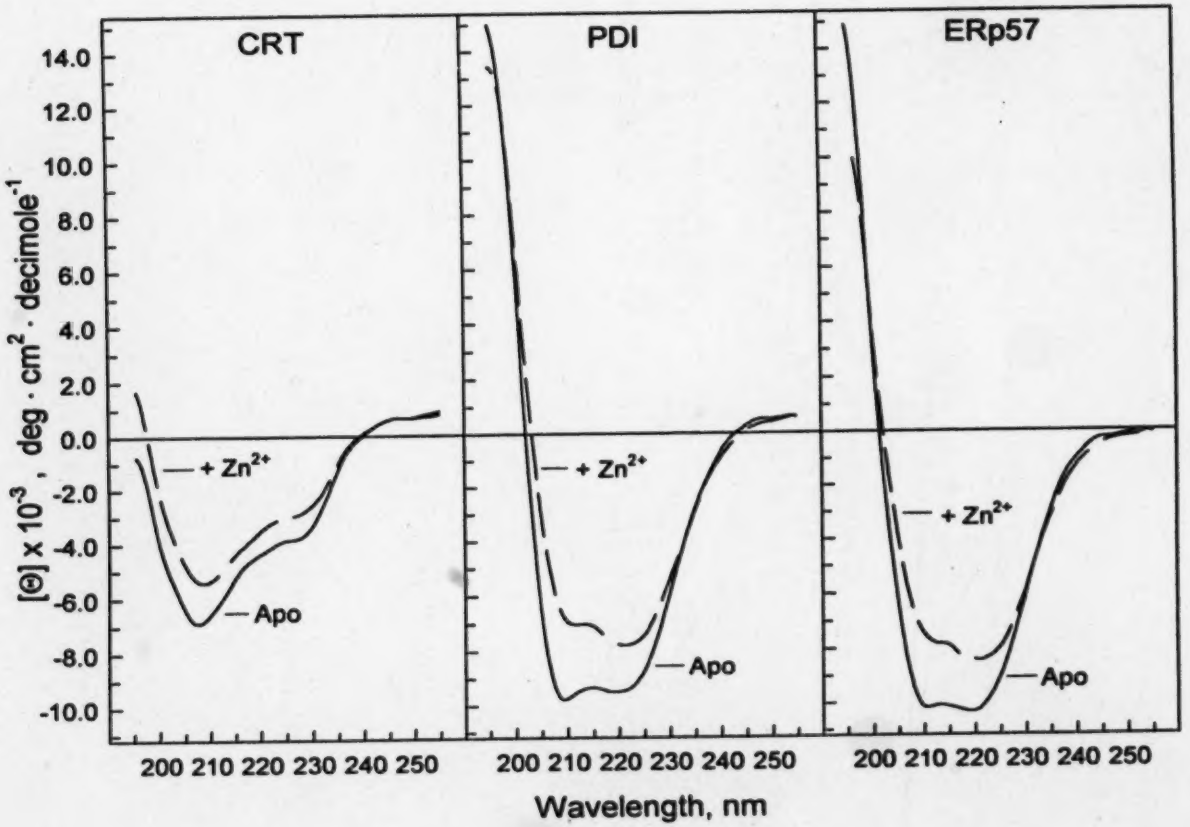
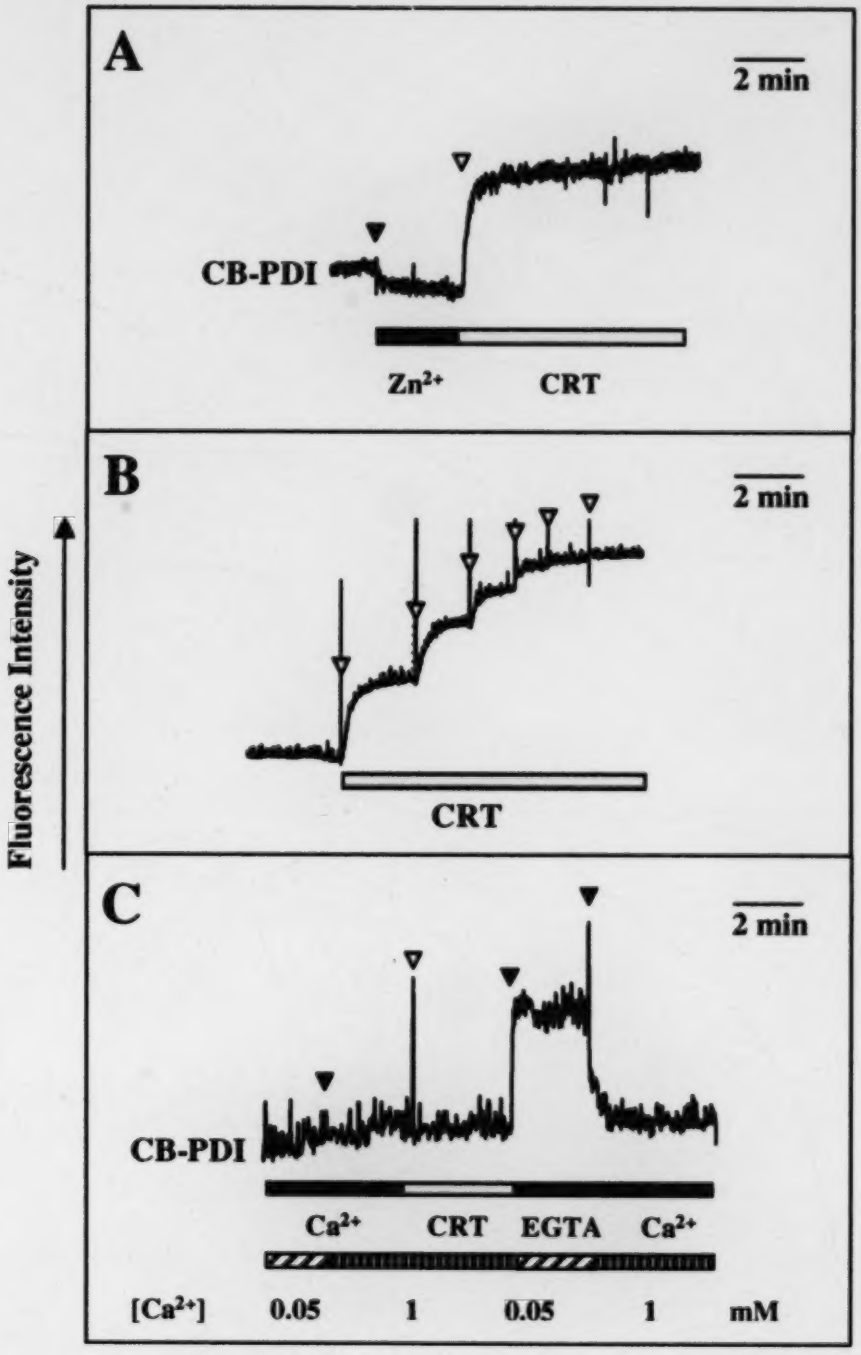

CRT
PDI
ERp57
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
-2.0
-4.0
-6.0
-8.0
-10.0
[Θ] x 10^-3, deg · cm^2 · decimole^-1
+ Zn2+
Apo
200 210 220 230 240 250
Wavelength, nm

Table 3-2: Provencher-Glocker secondary analysis of ERp57, calreticulin, PDI and complexes of calreticulin-ERp57 and calreticulin-PDI

Sample	α-Helix	β-Sheet	β-Turn	Remainder	Scale factor
ERp57 apo	0.25 (± 0.003)	0.37 (± 0.005)	0.12 (± 0.006)	0.26 (± 0.007)	1.035
ERp57 1 mM Ca^{2+}	0.25 (± 0.007)	0.35 (± 0.012)	0.14 (± 0.014)	0.26 (± 0.011)	1.027
ERp57 1 mM Zn^{2+}	0.08 (± 0.004)	0.40 (± 0.007)	0.22 (± 0.012)	0.30 (± 0.007)	0.971
Calreticulin apo	0.09 (± 0.005)	0.38 (± 0.008)	0.29 (± 0.009)	0.24 (± 0.008)	1.029
Calreticulin 1 mM Ca^{2+}	0.11 (± 0.005)	0.34 (± 0.008)	0.29 (± 0.009)	0.26 (± 0.008)	1.046
Calreticulin 1 mM Zn^{2+}	0.12 (± 0.003)	0.41 (± 0.005)	0.22 (± 0.006)	0.25 (± 0.003)	1.006
Complex apo	0.19 (± 0.006)	0.32 (± 0.009)	0.19 (± 0.009)	0.30 (± 0.012)	1.025
Cal complex apo	0.18 (± 0.002)	0.37 (± 0.005)	0.20 (± 0.005)	0.25 (± 0.004)	1.032
Delta	0.01	-0.05	-0.01	0.05	
Complex 1 mM Ca^{2+}	0.20 (± 0.003)	0.38 (± 0.005)	0.18 (± 0.005)	0.24 (± 0.011)	0.999
Delta	0.02	0.01	-0.20	-0.01	
Complex 1 mM Zn^{2+}	0.17 (± 0.003)	0.39 (± 0.006)	0.17 (± 0.006)	0.25 (± 0.004)	1.014
Delta	-0.01	0.02	-0.03	0.00	
PDI apo	0.30 (± 0.004)	0.34 (± 0.007)	0.15 (± 0.009)	0.21 (± 0.006)	1.005
PDI 2 mM Ca^{2+}	0.28 (± 0.005)	0.30 (± 0.009)	0.19 (± 0.012)	0.22 (± 0.008)	1.024
PDI 2 mM Zn^{2+}	0.21 (± 0.004)	0.34 (± 0.008)	0.21 (± 0.010)	0.24 (± 0.007)	1.023
Calreticulin apo	0.09 (± 0.00[illegible])	0.34 (± 0.009)	0.32 (± 0.011)	0.25 (± 0.007)	1.051
Calreticulin 2 mM Ca^{2+}	0.06 (± 0.005)	0.41 (± 0.007)	0.31 (± 0.009)	0.22 (± 0.005)	1.054
Calreticulin 2 mM Zn^{2+}	0.03 (± 0.008)	0.41 (± 0.013)	0.36 (± 0.015)	0.21 (± 0.007)	1.080
Complex apo	0.17 (± 0.008)	0.34 (± 0.014)	0.28 (± 0.016)	0.21 (± 0.013)	1.076
Cal complex apo	0.20 (± 0.004)	0.34 (± 0.007)	0.23 (± 0.009)	0.23 (± 0.006)	1.027
Delta	-0.03	0.00	0.05	-0.02	
Complex 2 mM Ca^{2+}	0.23 (± 0.002)	0.32 (± 0.004)	0.19 (± 0.004)	0.25 (± 0.002)	0.990
Delta	0.03	-0.02	-0.04	0.02	
Complex 2 mM Zn^{2+}	0.15 (± 0.005)	0.35 (± 0.009)	0.28 (± 0.010)	0.21 (± 0.009)	1.077
Delta	-0.05	0.01	0.05	-0.02	

Figure 3-5: Interaction between calreticulin and CB-PDI

CB-PDI was generated as described under "Methods", Chapter Two. **(A)** – effects of 500 μM Zn^{2+} and purified calreticulin on the fluorescence intensity of CB-PDI; **(B)** – effects of increased levels of calreticulin on fluorescence of CB-PDI; **(C)** - Ca^{2+} dependence of interaction between calreticulin and CB-PDI. Levels of 50 μM or 1 mM free Ca^{2+}concentration were maintained by the addition of 1 mM EGTA. Bars represent the duration of incubation with ions or purified proteins. Arrowheads depict the time of addition.


A
2 min
CB-PDI
Zn2+
CRT
B
2 min
Fluorescence Intensity
CRT
C
2 min
CB-PDI
Ca2+
CRT
EGTA
Ca2+
[Ca2+]
0.05
1
0.05
1
mM

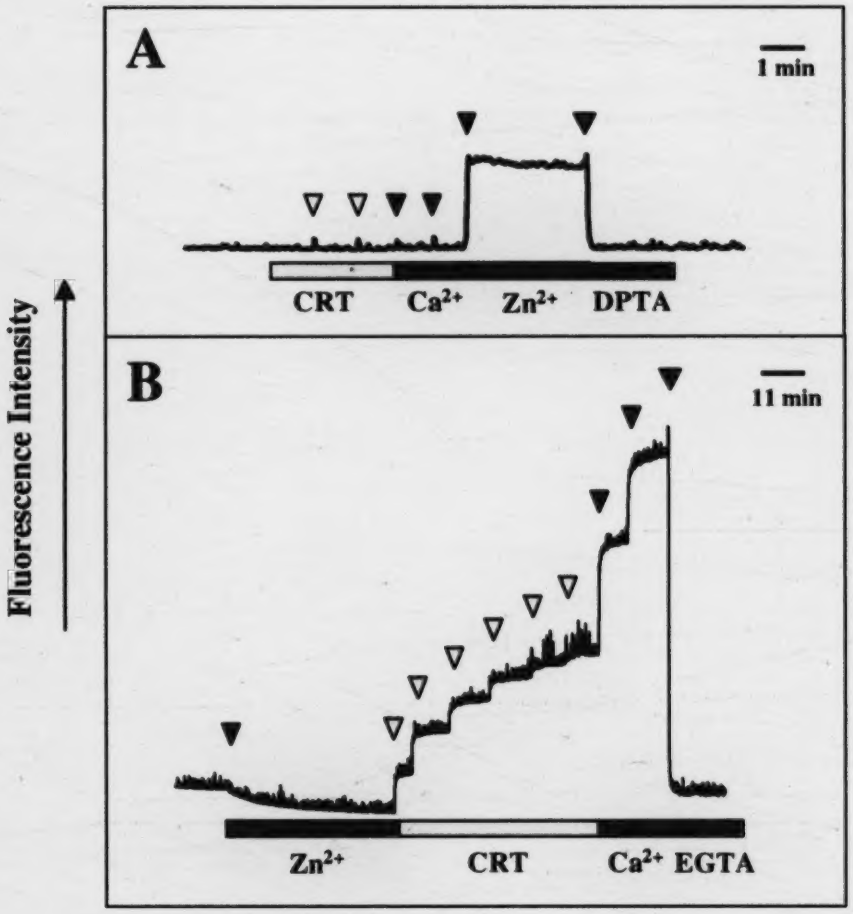
conformational changes in the protein followed by addition of the purified pancreatic calreticulin (Fig. 3-5A). Figure 3-5A shows that calreticulin induced a very rapid increase in fluorescence intensity of CB-PDI indicative of protein-protein interaction-induced conformational changes in the protein. The calculated initial rates of interaction between calreticulin and PDI was 21.4 ± 0.6 units/second (mean ± SE, n=3), whereas the time constant of the process was 0.16 ± 0.01 second (mean ± SE, n=3). Figure 3-5B illustrates that the effect was saturable with respect to calreticulin and did not require the presence of Zn^{2+} (Fig. 3-5B). The calculated ratio of binding of calreticulin: PDI was 1:1. Using the luminal domain of calnexin as a control, we demonstrated that the saturation of CB-PDI was specific to calreticulin. Figure 3-5C reveals that, in agreement with our earlier observations (Baksh *et al.*, 1995a), calreticulin did not interact with PDI in the presence of 1 mM Ca^{2+}. Hormone-stimulated Ca^{2+} depletion results in lowering of the ER luminal free Ca^{2+} concentration below 100 μM (Montero *et al.*, 1995; Miyawaki *et al.*, 1997; Meldolesi and Pozzan, 1998a). We have tested, therefore, if calreticulin and CB-PDI will interact under the conditions of Ca^{2+} store depletion. Addition of EGTA to lower free Ca^{2+} concentration to 50 μM restored interaction between calreticulin and CB-PDI as revealed by changes in the fluorescence intensity of the CB-labeled protein (Fig. 3-5C). Re-addition of Ca^{2+} (to 1 mM) rapidly reduced the fluorescence to the original level indicative of dissociation of the protein complex (Fig. 3-5C). Ca^{2+} titration experiments revealed that the EC_{50} for Ca^{2+} for calreticulin-PDI dissociation was approximately 110 ± 15 μM (mean ± SE, n=3). Thus, there is a saturable, rapidly reversible and Ca^{2+}-dependent (under physiologically relevant Ca^{2+} concentrations) interaction between calreticulin and PDI.

Interactions between calreticulin and ERp57

Several groups have shown that ERp57, similar to calreticulin and calnexin, is involved in chaperoning of glycoproteins (Elliott *et al.*, 1997; Hughes and Cresswell, 1998; Lindquist *et al.*, 1998; Morrice and Powis, 1998; Oliver *et al.*, 1997). Furthermore, Zapun *et al* (Zapun *et al.*, 1998) reported functional interactions between calreticulin and ERp57. However, direct interaction between calreticulin and ERp57 has not yet been documented. We utilized CB-ERp57 to investigate binding of calreticulin to ERp57 and the role of Ca^{2+} in these protein-protein interactions. Figure 3-6A shows that addition of calreticulin to CB-ERp57 either in the presence or absence of Ca^{2+} had no effect on fluorescence intensity of CB-ERp57, suggesting that under these conditions the proteins may not have interacted. Since Zn^{2+} induced conformational changes in ERp57 (Figure 3-3C), we tested if the presence of Zn^{2+} had any effect on the protein interaction with calreticulin. Zn^{2+} induced a *decrease* in fluorescence intensity of CB-ERp57 alone (Fig. 3-3C). In contrast, addition of calreticulin to CB-ERp57 in the presence of Zn^{2+} resulted in an *increase* in the fluorescence intensity (Fig. 3-6A) indicating that conformational changes in ERp57 were required to initiate its interaction with calreticulin. Chelating of Zn^{2+} with DTPA resulted in a decrease in the intensity of fluorescence, indicative of dissociation of the protein complex (Fig. 3-6A). The initial rate of this interaction was 23.4 ± 0.4 units/sec (mean ± SE, n=3) and it was similar to that observed for calreticulin and PDI interaction (21.4 ± 0.6 units/sec; mean ± SE, n=3). The time constant of interaction between ERp57 and calreticulin (1.4 ± 0.1 sec; mean ± SE, n=3) was approximately 9 fold higher than between PDI and calreticulin. Figure 3-6B illustrates that changes in the fluorescence intensity of CB-ERp57 were saturable with respect to

Figure 3-6: Interaction between calreticulin and CB-ERp57

ERp57 was labeled with CB as described under "Methods", Chapter Two. **(A)** – effects of calreticulin, 1 mM Ca^{2+} and 1 mM Zn^{2+} on fluorescence intensity of CB-ERp57. DTPA was added to chelate Zn^{2+}. **(B)** – effects of increasing levels of calreticulin and Ca^{2+} on fluorescence intensity of CB-ERp57. Bars represent the duration of incubation with ions or purified proteins. Arrowheads depict the time of addition.


A
1 min
CRT
Ca2+
Zn2+
DPTA
B
11 min
Zn2+
CRT
Ca2+ EGTA
Fluorescence Intensity

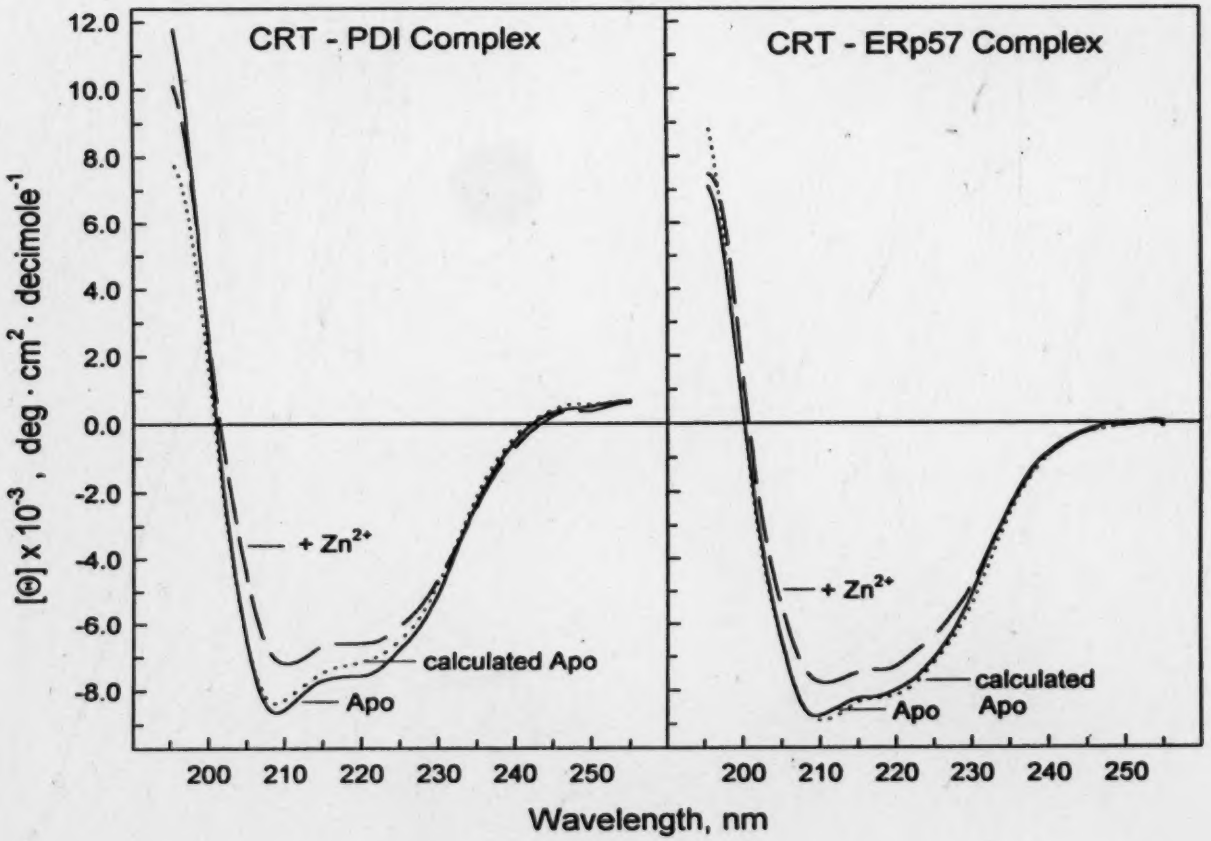
calreticulin. Ca^{2+} did not affect initial interactions between ERp57 and calreticulin (Fig. 3-6A). Surprisingly, Ca^{2+} had an effect on already formed calreticulin-ERp57 protein complex. Fluorescence intensity of CB-ERp57 and calreticulin complex was increased by addition of 1 mM Ca^{2+} (Fig. 3-6B). Titration of calreticulin binding to ERp57 in the presence or absence of Ca^{2+} revealed that Ca^{2+} had no effect on the calreticulin affinity to bind ERp57 but only on the fluorescence intensity of the complex. The calculated ratio of binding of calreticulin: ERp57 was 1:1. We concluded that Ca^{2+} induced new conformational change in the calreticulin-ERp57 complex without recruiting additional molecules of calreticulin. The Ca^{2+}-dependent enhancement of fluorescence was reversed by addition of EGTA (Fig. 3-6B). Analysis of the Ca^{2+}-dependance of this process revealed that the EC_{50} for Ca^{2+} was approximately 400 ± 30 μM (mean ± SE, n=3). Thus, interactions between calreticulin and ERp57 initially required a Zn^{2+}-dependent but Ca^{2+}-independent conformational change in the ERp57. The properties of the complex were further influenced by increased Ca^{2+} concentration.

Interaction between calreticulin and PDI and ERp57 induce conformational changes in protein complexes

We used CD analysis to further investigate interaction between calreticulin and ERp57 and PDI. Figure 3-7 represents the observed and calculated CD spectra of complexes between calreticulin and PDI and between calreticulin and ERp57. Their secondary structural analysis is indicated in Table 3-2. In the absence of Ca^{2+} and Zn^{2+} the ERp57 complex with calreticulin showed comparable observed and calculated spectra, the latter estimated by weight percentage of each component of the complex. This would imply that under these conditions there was an interaction with minimal attendant conformational change. In agreement with our fluorescence measurements,

Figure 3-7: CD analysis of protein complexes

Representative CD spectra of calreticulin, PDI and ERp57 in the absence (——) and presence of Zn^{2+} (- - - -). Also included are the calculated spectra for the apo state of the complexes (·····). Kim Oikawa performed the CD analysis.


CRT - PDI Complex
CRT - ERp57 Complex
12.0
10.0
8.0
6.0
4.0
2.0
0.0
-2.0
-4.0
-6.0
-8.0
[Θ] x 10^-3, deg · cm^2 · decimole^-1
+ Zn2+
calculated Apo
Apo
200
210
220
230
240
250
Wavelength, nm

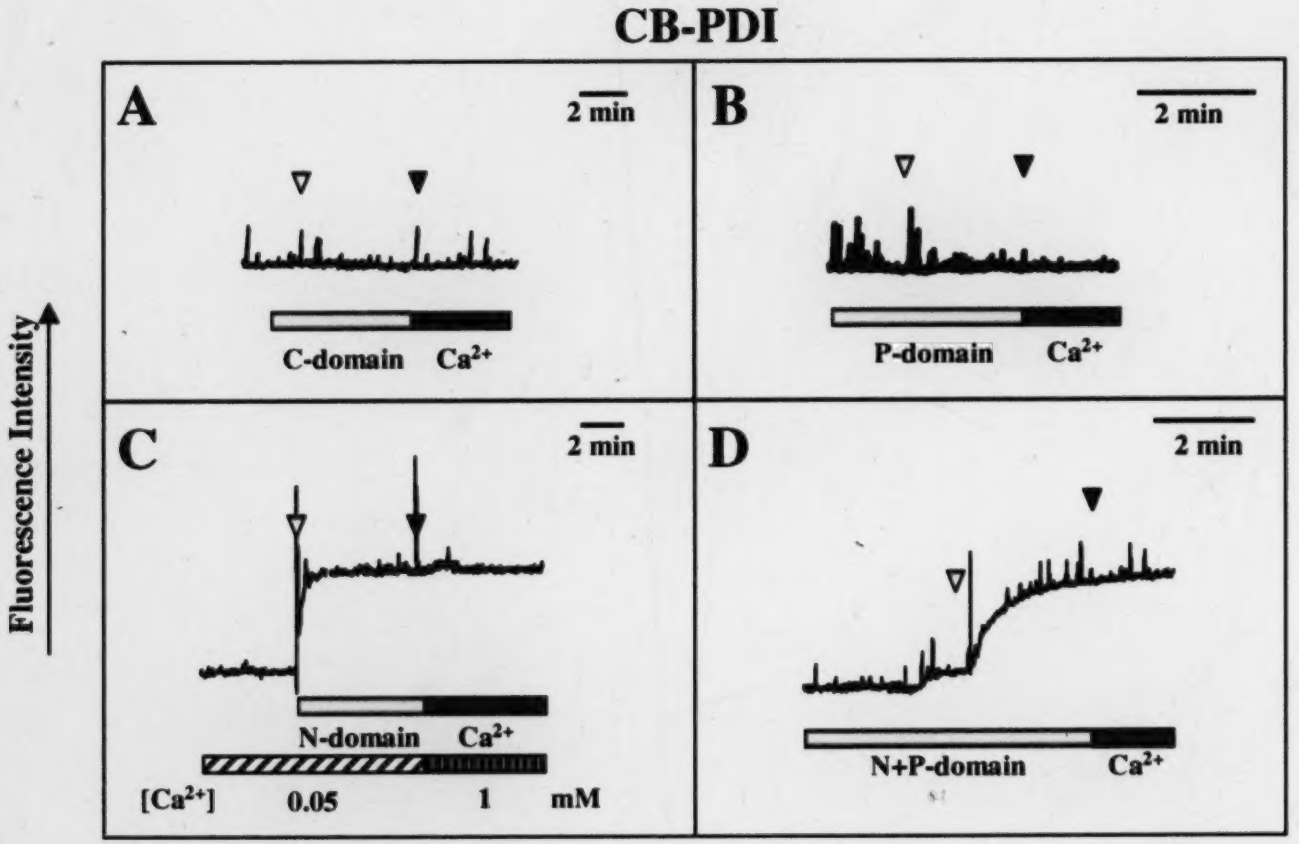
addition of Zn^{2+} to the mixture resulted in a larger change in the observed versus calculated spectra (Fig. 3-7 and Table 3-2). The PDI complex with calreticulin produced small changes for the observed apo and Ca^{2+} conditions relative to the calculated values, while Zn^{2+} once again produced the greatest change. Ca^{2+} resulted in a slight increase in α-helix while the apo and Zn^{2+} conditions showed a drop in α-helix upon Contin analysis.

A role of calreticulin domains in protein-protein interactions

In order to establish the specific region of calreticulin involved in the interaction with PDI and ERp57 we expressed calreticulin domains in *E. coli*. The following domains of calreticulin were used: the N-domain (the NH_2-terminal 182 amino acids of the protein), the P-domain (residues 182-273), the N+P-domain (residues 1-273) and the C-domain (residues 270-401) (Baksh and Michalak, 1991). Previously by ligand blotting, affinity chromatography and the yeast two hybrid system it was shown that PDI interacts with the N-domain and P-domain of calreticulin in the absence of Ca^{2+} (Baksh *et al.*, 1995a). Figure 3-8 shows that in the absence of Ca^{2+} the P-domain (Fig. 3-8B) and C-domain (Fig. 3-8A) of calreticulin had no effect on the intensity of fluorescence of CB-PDI suggesting that under those conditions these domains did not interact with calreticulin. Addition of the N-domain (Fig. 3-8C) or N+P-domain (Fig. 3-8D) of calreticulin to CB-PDI in the presence of 50 μM Ca^{2+} induced changes in the fluorescence intensity of the CB-PDI indicative of protein-protein interaction similar to those observed in the presence full-length calreticulin (compare Fig. 3-8C and 3-5C). Increasing Ca^{2+} concentration up to 1 mM had no effect on fluorescence intensity of the

Figure 3-8: (A-D) Effects of calreticulin domains on fluorescence intensity of CB-PDI and CB-ERp57

Calreticulin domains were expressed in *E. coli* and purified as described under "Methods", Chapter Two. CB labeling of PDI and ERp57 was carried out as described under "Methods", Chapter Two. The effect of the C-domain (**A, E**), P-domain (**B, F**), N-domain (**C, G**) and N+P-domain (**D, H**) of calreticulin on fluorescence intensity of CB-PDI (**A-D**) and ERp57 (**E-H**) was measured as described under "Methods, Chapter Two". Zn^{2+} or Ca^{2+} were added when indicated by the arrowheads. Bars represent the duration of incubation with ions or purified proteins as indicated.

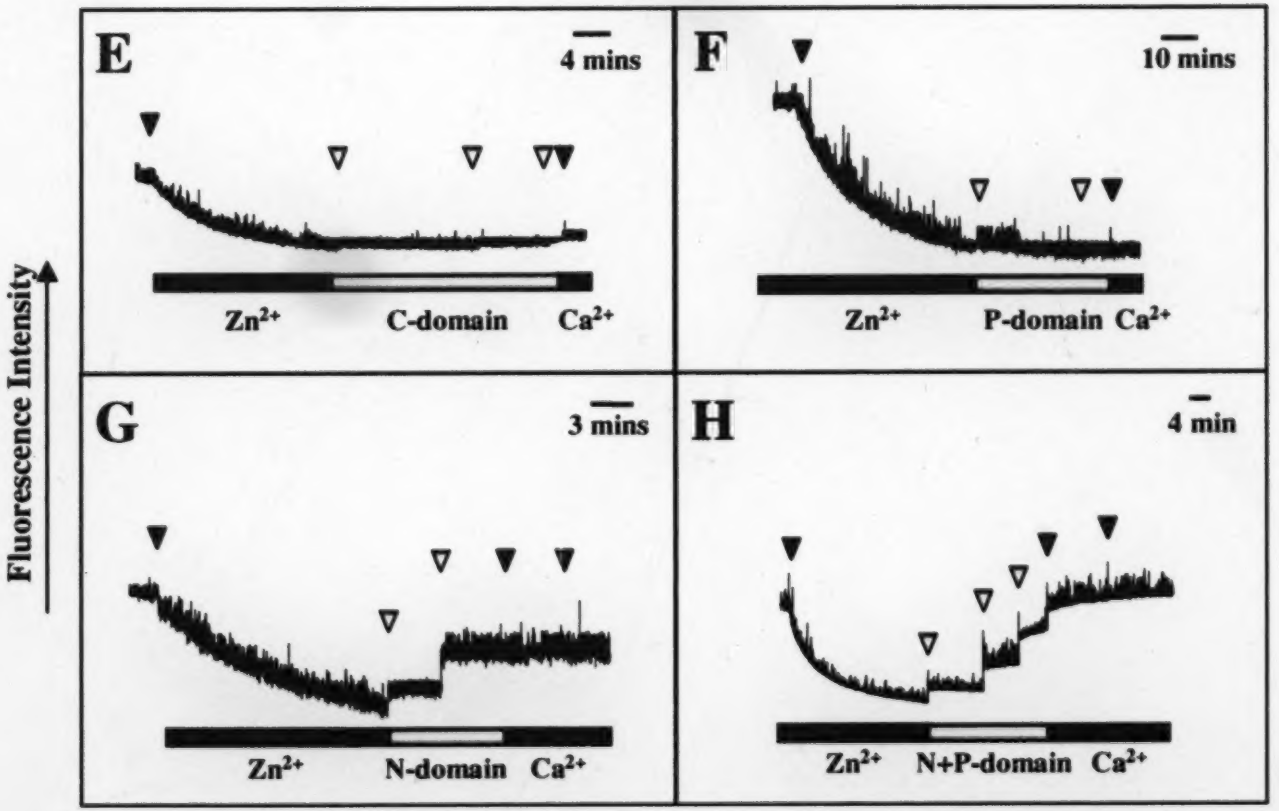

CB-PDI
A
2 min
C-domain
Ca2+
B
2 min
P-domain
Ca2+
C
2 min
N-domain
Ca2+
[Ca2+]
0.05
1
mM
D
2 min
N+P-domain
Ca2+
Fluorescence Intensity

CB-PDI and N-domain (Fig. 3-8C) or CB-PDI and N+P-domain complexes (Fig. 3-8D). This is in contrast to the Ca^{2+}-dependent dissociation of the full-length calreticulin and CB-PDI complex (Fig. 3-5C). We concluded that Ca^{2+} binding to the C-domain of calreticulin was responsible for Ca^{2+}-dependent dissociation of the calreticulin/PDI complex. Thus, the N-domain of calreticulin is required and sufficient for interaction between calreticulin and PDI. The C-domain of the protein plays a role in dissociation of the protein complex.

Next we examined the role of calreticulin domains in interactions with ERp57. Figure 3-8E and Figure 3-8F shows that the P-domain and C-domain of calreticulin did not have any effect on the fluorescence intensity of CB-ERp57 regardless of the conditions used. However, addition of the N-domain or N+P-domain of the protein resulted in an increase in the intensity of fluorescence of CB-ERp57 suggesting that the two proteins interact (Fig. 3-8G and 3-8H). The effect of the N-domain or N+P-domain was identical to that of the full-length calreticulin (compare Fig. 3-8G, 3-8H and Fig. 3-6B). Since the P-domain alone did not have any effect on the CB-ERp57 fluorescence we concluded that the N-domain of calreticulin interacted with ERp57. Similar to full-length calreticulin, initial interaction between the N-domain of calreticulin and CB-ERp57 was Ca^{2+}-independent and required the presence of Zn^{2+} (Fig. 3-8G and 3-8H). Importantly, Ca^{2+} had no effect on the N-domain and CB-ERp57 complex (Fig. 3-8G and 3-8H). This is in contrast to full-length calreticulin (compare Fig. 3-8G, 3-8H and 3-6B). We concluded that the N-domain of calreticulin is essential for interaction between calreticulin and ERp57. The P-domain does not participate in the interaction. Once

Figure 3-8: (E-F) Effects of calreticulin domains on fluorescence intensity of CB-PDI and CB-ERp57

Calreticulin domains were expressed in *E. coli* and purified as described under "Methods", Chapter Two. CB labeling of PDI and ERp57 was carried out as described under "Methods", Chapter Two. The effect of the C-domain (**A, E**), P-domain (**B, F**), N-domain (**C, G**) and N+P-domain (**D, H**) of calreticulin on fluorescence intensity of CB-PDI (**A-D**) and ERp57 (**E-H**) was measured as described under "Methods, Chapter Two". Zn^{2+} or Ca^{2+} were added when indicated by the arrowheads. Bars represent the duration of incubation with ions or purified proteins as indicated.


CB-ERp57
E
4 mins
Zn2+
C-domain
Ca2+
F
10 mins
Zn2+
P-domain
Ca2+
G
3 mins
Zn2+
N-domain
Ca2+
H
4 min
Zn2+
N+P-domain
Ca2+
Fluorescence Intensity

again these results suggest that the C-domain of the protein plays a role in Ca^{2+}-dependent augmentation of the calreticulin-ERp57 complex.

Discussion

In this study we utilized CB, a fluorescent dye, to investigate the dynamics of the interactions between calreticulin and two related ER membrane chaperones: PDI and ERp57. These interactions are modulated by changes in protein conformation and by fluctuations in free Ca^{2+} concentrations reminiscent of emptying and refilling of the ER Ca^{2+} stores. Calreticulin interacts with PDI at low Ca^{2+} concentration (<100 μM) but the protein complex dissociates upon increased Ca^{2+} concentration (>400 μM). Formation of the ERp57-calreticulin complex was initiated by a Ca^{2+}-independent conformational change in ERp57 followed by a Ca^{2+}-dependent conformational change in the complex. Ca^{2+} binding to the C-domain of calreticulin is responsible for the dissociation of the calreticulin–PDI complex and for the modulation of the interaction between calreticulin and ERp57 suggesting that this domain of the protein may play a role of a Ca^{2+} "sensor" for ER membrane chaperones. These results suggest changes in the free ER luminal Ca^{2+} concentration may be important for the regulation of these chaperone-chaperone interactions.

CB has been used previously as a fluorescent indicator for labeling of protein, including antibodies (Whitaker *et al.*, 1991). However, to our knowledge, this is the first time the dye has been utilized to study changes in protein conformation due to either ion binding or protein-protein interactions. This property of the dye is likely a result of different behaviors of CB in solvents (environments) of various polarities. Indeed, we

show that the fluorescence intensity of CB changes with the exposure to hydrophobic or hydrophilic environments suggesting that exposure of the dye to different regions in proteins may also lead to changes in its fluorescence intensity. For example, changes in fluorescence intensity of CB-PDI and CB-ERp57 due to Zn^{2+} binding or interaction with calreticulin correspond with changes in their CD spectra. It is conceivable that the dye may be used to study other protein-protein interactions involving conformational changes in proteins.

One of the most important observations in this study is that the interactions between calreticulin, PDI and ERp57 are regulated by fluctuation of Ca^{2+} concentration. For example calreticulin interacts with PDI only when the Ca^{2+} concentration is below 100 μM, a Ca^{2+} concentration found upon emptying of the Ca^{2+} stores (Montero *et al.*, 1995; Miyawaki *et al.*, 1997; Meldolesi and Pozzan, 1998a). The complex dissociates at increased Ca^{2+} concentration (>400 μM) a concentration common for refilled Ca^{2+} (Montero *et al.*, 1995; Miyawaki *et al.*, 1997; Meldolesi and Pozzan, 1998a). Ca^{2+} is released from the ER *via* $InsP_3$ receptor/Ca^{2+} channel and it is taken up by Ca^{2+}-ATPase (SERCA) (Pozzan *et al.*, 1994). In stimulated cells this is a rapid process resulting in continuous changes in the levels of free ER luminal Ca^{2+} concentrations (Meldolesi and Pozzan, 1998a). We show that interaction between calreticulin and PDI and calreticulin and ERp57 are also very rapid with time constants of 0.16 ± 0.01 and 1.4 ± 0.1 sec, respectively. Therefore, as far as the kinetics of these protein-protein interactions are concerned, they are physiologically relevant and capable of responding to rapid fluctuations in the luminal Ca^{2+} concentrations.

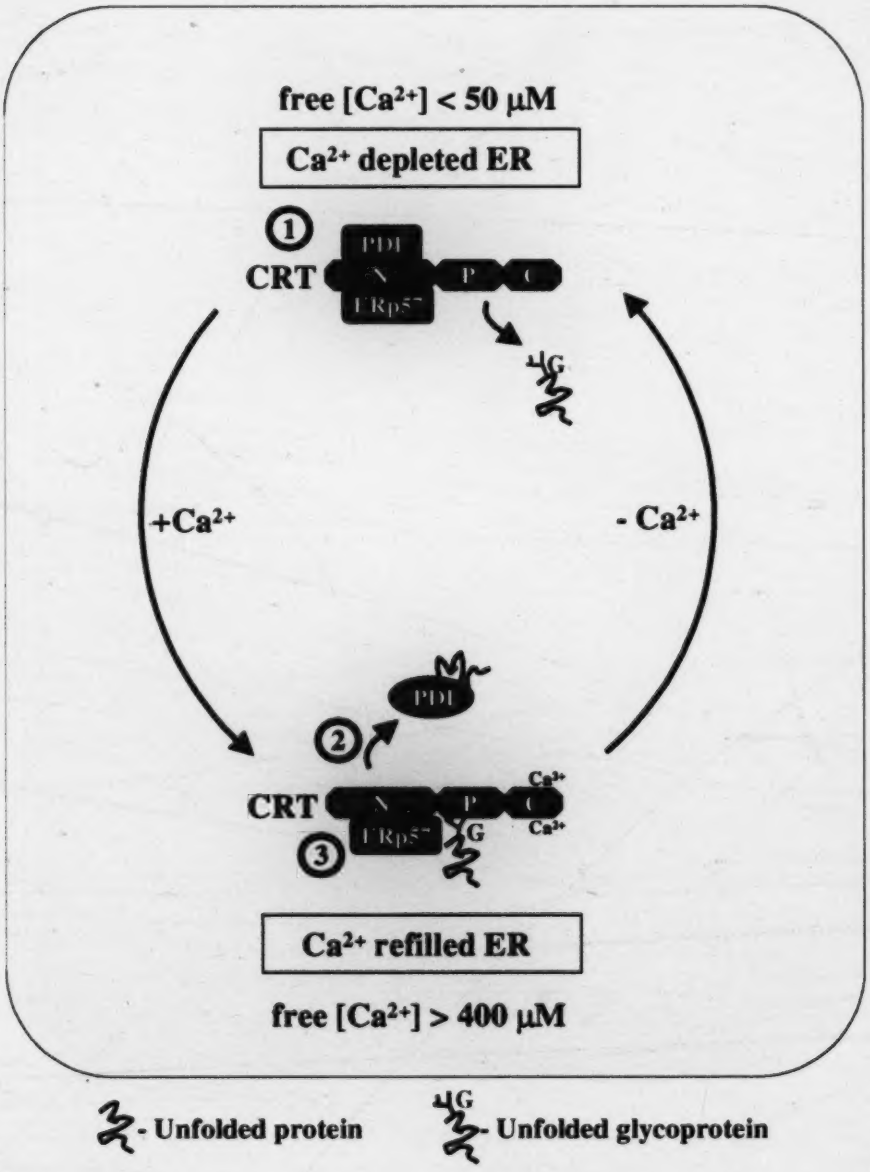
Interactions between calreticulin and CB-PDI is restricted to the N-terminal region (N-domain) of calreticulin, but Ca^{2+}-sensitivity of this interaction is confined to the C-terminal, high capacity Ca^{2+} binding region (C-domain) of the protein. We also established that ERp57 forms protein complexes with calreticulin but unlike the calreticulin-PDI complex, binding of calreticulin to ERp57 is initiated by a Ca^{2+}-independent, conformational change in ERp57 followed by a Ca^{2+}-dependent modulation of the complex. Once again the N-domain of calreticulin plays a key role in this protein-protein interaction and the C-domain is responsible for the Ca^{2+}-dependent enhancement of the complex. In support of our results, High *et al* (Oliver *et al.*, 1999) also showed that ERp57 forms distinct complexes with both calreticulin and calnexin in the ER lumen and when the proteins were mixed in solution. They also, like us, show a direct interaction between ERp57 and calreticulin and calnexin and that this interaction does not require the presence of glycoprotein substrates (Oliver *et al.*, 1999). The N-domain of calreticulin is the most conserved region in the protein with over 70% amino acid similarity between human and higher plants (Michalak, 1995). One important function of the N-domain of calreticulin may be formation of specific complexes between ER luminal chaperones. The C-domain of the protein may play a Ca^{2+}-dependent regulatory role in these protein-protein interactions. The C-domain of calreticulin is the least conserved region of the protein, and in some organisms it is even missing (Michalak, 1995). While in all organisms the N-domain of calreticulin may be responsible for formation of protein complexes between different ER chaperones not all of them will have a Ca^{2+}-dependent (C-domain-dependent) regulation of these complexes.

What is the physiological relevance of these specific protein-protein interactions? Figure 3-9 shows a proposed model for a role of calreticulin and Ca^{2+} in controlling interactions between ER luminal proteins and in regulation of their chaperone function. Under the conditions of empty Ca^{2+} stores (Fig. 3-9 **(1)**), when free Ca^{2+} concentration is below 100 μM (Miyawaki *et al.*, 1997), calreticulin will form tight complexes with PDI. This protein-protein interaction may result in inhibition of PDI activity (Baksh *et al.*, 1995a; Zapun *et al.*, 1998). Under low Ca^{2+} conditions unfolded proteins may be released from PDI to enable them to interact with other chaperones to continue the process of folding and QC. This may allow a flux of proteins undergoing a folding process, from one chaperone to the other. Calreticulin is estimated to consist of ~ 0.5% of total cellular protein (Khanna and Waisman, 1986), while PDI is estimated at ~ 0.8% of total cellular protein (Ferrari and Söling, 1999). However, upon examination of pancreatic microsomes by SDS-PAGE, the abundance of calreticulin to PDI is approximately equal, with slightly more calreticulin present (Michalak *et al.*, 1991). It is well established that Ca^{2+} depletion of the ER Ca^{2+}stores by thapsigargin leads to induction of the unfolded protein pathway (Li *et al.*, 1993; Llewellyn *et al.*, 1996; Mori *et al.*, 1993; Nguyen *et al.*, 1996; Pahl and Baeuerle, 1997; Waser *et al.*, 1997). Results of our work indicate that prolonged Ca^{2+} depletion of the ER may result in a massive release of unfolded proteins from PDI and calreticulin activating the UPR pathway, without actually changing a total concentration of unfolded proteins.

Under the low Ca^{2+} conditions calreticulin will also interact with ERp57 (Fig. 3-9 **(1)**). These complexes may not be functional, as far as lectin-like chaperoning is

Figure 3-9: Ca^{2+}-dependent interactions between calreticulin, PDI, ERp57 and unfolded glycoproteins.

(1) At concentrations of Ca^{2+} measured during agonist-dependent Ca^{2+}-depletion of the ER (<50 μM), PDI and ERp57 interact with calreticulin. **(2)** At concentrations of Ca^{2+} measured when ER Ca^{2+} stores are full (>400 μM), PDI and calreticulin do not associate and PDI carries out its chaperone functions alone or in co-ordination with other chaperones. **(3)** Increased free $[Ca^{2+}]_{ER}$ promote binding of monoglucosylated glycoproteins to the P-domain of calreticulin (and the central domain of calnexin). Under these conditions calreticulin and ERp57 are associated, forming fully functional chaperoning complexes. G, glucose residue. Reprinted from *TiBS, 25,* Corbett, E.F., Michalak, M. Calcium, a signaling molecule in the endoplasmic reticulum. 307-311. © 2000, with permission from *Elsevier Science.*


free [Ca2+] < 50 μM
Ca2+ depleted ER
1
CRT
PDI
N
P
C
ERp57
G
+Ca2+
- Ca2+
PDI
2
CRT
Ca2+
Ca2+
3
Ca2+ refilled ER
free [Ca2+] > 400 μM
- Unfolded protein
G
- Unfolded glycoprotein

concerned, because at a low Ca^{2+} concentration calreticulin displays a very weak, if any, binding to monoglucosylated glycoproteins (Vassilakos *et al.*, 1998). However, these complexes may be functional as far as 'true' molecular chaperoning is concerned, as calreticulin is now known to interact with non-glycosylated substrates (Saito *et al.*, 1999). Refilling of Ca^{2+} stores *via* function of SERCA will result in significant changes in the dynamics of luminal ER proteins. Under these conditions free Ca^{2+} concentration will raise above 400 μM (Miyawaki *et al.*, 1997) followed by a rapid dissociation of calreticulin from PDI (Fig. 3-9 **(2)**). Since, under these conditions PDI does not associate with calreticulin (or calnexin) it is likely that the protein may play an important role in disulfide bond formation of newly synthesized proteins that are not glycosylated. Importantly, elevations in the free Ca^{2+} concentration in the lumen of the ER will promote calreticulin (and calnexin) lectin-like activity and their interaction with monoglucosylated glycoproteins (Fig. 3-9). Calreticulin will recruit ERp57 to provide an "attachment" site for the protein to chaperone (disulfide bond formation) newly synthesized glycoproteins (Fig. 3-9 **(3)**). ERp57 chaperone activity is greatly increased while complexed with calreticulin (Zapun *et al.*, 1998). Under these conditions other ER luminal chaperones may also be recruited to further assist in proper folding of newly synthesized glycoproteins. It would be interesting to see the combined effects of calreticulin and calnexin on functional studies on ERp57 and PDI. One significant finding is that the high capacity Ca^{2+} binding site (C-domain) of calreticulin may play a role of a Ca^{2+} "sensor" for these protein-protein interactions in response to continuous fluctuations of ER luminal Ca^{2+} concentrations. The C-domain of calreticulin is expected to bind Ca^{2+} only in fully re-filled Ca^{2+} stores but not under the ER Ca^{2+} depletion

conditions (Baksh and Michalak, 1991). It is likely, therefore, that calreticulin, *via* its Ca^{2+} binding ability, may play multiple roles in the lumen of the ER: regulation of free Ca^{2+} concentration and Ca^{2+}-dependent modulation of chaperone function of other ER luminal proteins such as PDI and ERp57. It has previously been reported that the function of other chaperones may be sensitive to fluctuations in the ER luminal Ca^{2+}. At low Ca^{2+} concentrations in the lumen of the ER the activity of BiP, another ER luminal chaperone, is also inhibited (Ivessa *et al.*, 1995). Protein synthesis, glycoprotein processing and transport competence are also blocked under the conditions of ER Ca^{2+} depletion (Srivastava *et al.*, 1995; Brostrom and Brostrom, 1998; Reilly *et al.*, 1998). Our work suggests that calreticulin may play a key role in the control of these processes.

Calreticulin, from the lumen of the ER, may regulate Ca^{2+} levels in the lumen of ER *via* potential interactions with the ER Ca^{2+}-ATPase (SERCA) and/or the $InsP_3$ receptor (Camacho and Lechleiter, 1995; John *et al.*, 1998). Favre *et al* (Favre *et al.*, 1996) demonstrated a highly supralinear feed-back inhibition of Ca^{2+} uptake *via* SERCA and postulated existence of an ER luminal molecule(s) which may regulate SERCA activity (John *et al.*, 1998). Calreticulin may be a potential ER luminal candidate protein that modulates SERCA activity. Furthermore, calreticulin, from the lumen of the ER, affects steroid sensitive gene expression (Michalak *et al.*, 1996), cell adhesiveness (Opas *et al.*, 1996a) and store-operated Ca^{2+} influx (Bastianutto *et al.*, 1995; Mery *et al.*, 1996). Results of this work support our earlier hypothesis (Krause and Michalak, 1997) and suggest that fluctuation of the ER luminal Ca^{2+} concentration will regulate the free concentration of calreticulin in the lumen of the ER. For example, at high luminal Ca^{2+} concentration levels of free calreticulin, in the lumen of the ER, will be significantly

increased. Thus, Ca^{2+}-dependent changes in the free calreticulin may play a role in modulation of a variety of calreticulin-dependent signals, including gene expression and Ca^{2+} homeostasis.

In summary, results of this work show that calreticulin appears to be one of the key players of the chaperone network in association with calnexin, PDI and ERp57. Furthermore, present work indicates that calreticulin may play a role of Ca^{2+} "sensor" for these chaperones. The protein modulates Ca^{2+}-dependent formation and maintenance of the structural and functional complexes between different ER luminal proteins involved in a variety of steps during protein synthesis, folding and posttranslational modification. It is conceivable that other chaperones may form similar functional complexes with calreticulin and that these interactions may be controlled by hormone-dependent fluctuations of free $[Ca^{2+}]_{ER}$.

Chapter Four

The Conformation of calreticulin is influenced by the Endoplasmic Reticulum luminal environment

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Introduction

The lumen of the ER is a dynamic environment perfectly designed for the task of protein folding. It contains a high concentration of Ca^{2+}-binding chaperones and an optimal concentration of ions and nucleotides for protein folding, modification and assembly. Several studies, including our own, have indicated that changes in the concentration of Ca^{2+} in the ER lumen $[Ca^{2+}]_{ER}$ affect many ER functions. These include the synthesis, folding, post-translational modification and secretion of proteins, protein conformation, and chaperone-chaperone or chaperone-substrate interactions (Booth and Koch, 1989; Lodish and Kong, 1990; Sambrook, 1990; Lodish *et al.*, 1992; Baksh *et al.*, 1995a; Meldolesi, 1998; Meldolesi and Pozzan, 1998b; Vassilakos *et al.*, 1998; Corbett *et al.*, 1999). The ER lumen also contains ATP, which is required to support the correct folding and formation of disulfide bonds in many proteins (Dorner *et al.*, 1990; Braakman *et al.*, 1992; Dorner and Kaufman, 1994). An ATP transporter has been identified in the ER (Clairmont *et al.*, 1992; Berninsone and Hirschberg, 1998) and the ER proteins PDI and BiP are known to bind/utilize ATP in their chaperone action (Munro and Pelham, 1986; Flynn *et al.*, 1989; Wei *et al.*, 1995; Wei and Hendershot, 1995; Guthapfel *et al.*, 1996).

Calreticulin is one of the major Ca^{2+} binding proteins in the lumen of the ER. The protein can be divided into three main structural regions (Michalak *et al.*, 1999). The highly conserved N-domain binds Zn^{2+} with high capacity (Baksh *et al.*, 1995b), whereas the proline rich P-domain binds Ca^{2+} with high affinity (Baksh and Michalak, 1991). The C-domain, which may play a role in the Ca^{2+} storage function of calreticulin, binds Ca^{2+} with high capacity (Baksh and Michalak, 1991). In the lumen of the ER the protein

functions as a chaperone and a modulator of Ca^{2+} homeostasis (Michalak *et al.*, 1999). Calreticulin binds monoglucosylated oligosaccharides in a Ca^{2+}-dependent manner (Vassilakos *et al.*, 1998; Michalak *et al.*, 1999). Calreticulin also interacts with misfolded proteins and both ATP and Zn^{2+} have been shown to enhance this interaction (Ihara *et al.*, 1999; Saito *et al.*, 1999). Importantly, calreticulin responds to fluctuating Ca^{2+} levels in the lumen of the ER by modulating its ability to interact with other ER proteins and substrates (Chapter Three, (Corbett *et al.*, 1999). We have suggested that the binding of Ca^{2+} to the C-domain of calreticulin may allow it to function as a "Ca^{2+} sensor" for other ER chaperones (Chapter Three, (Corbett *et al.*, 1999). However, the precise effects of ER constituents such as Ca^{2+}, Zn^{2+}, nucleotides and other proteins, on the structure of calreticulin, remain to be determined.

As a way to provide a better understanding of the molecular mechanism underlying the chaperone function of calreticulin, we have focused our efforts on characterizing aspects of its three-dimensional structure. Using a series of biochemical and biophysical approaches we analyzed the three-dimensional structure of calreticulin. We investigated the effect of changes in the ER luminal environment (specifically Ca^{2+}, Zn^{2+}, ATP) on conformational changes in calreticulin. We also examine how interactions of PDI and ERp57 affect the conformation of calreticulin.

Results

Ca^{2+}-dependent conformational changes in calreticulin

In response to agonist stimulation of cells, the $[Ca^{2+}]_{ER}$ undergoes dynamic changes (Meldolesi and Pozzan, 1998a). In a steady-state situation, the $[Ca^{2+}]_{ER}$ is in

excess of 400 μM. After agonist-evoked Ca^{2+} release from the ER, the $[Ca^{2+}]_{ER}$ is reduced to <50 μM (Miyawaki *et al.*, 1997). Therefore, upon agonist stimulation, Ca^{2+}-binding proteins in the lumen of the ER, including calreticulin, experience frequent changes in Ca^{2+} concentration. To evaluate the importance of $[Ca^{2+}]_{ER}$ in calreticulins function, we investigated the effect of Ca^{2+} on calreticulins sensitivity to protease digestion. Large quantities of calreticulin were expressed in *Pichia* and purified (Andrin *et al.*, 2000). The purified protein was digested with trypsin (1:100 trypsin:calreticulin) over a range of physiologically relevant Ca^{2+} concentrations, from low (0-100 μM representing empty Ca^{2+} stores) to high (500-1000 μM representing full Ca^{2+} stores) (Meldolesi and Pozzan, 1998a). Figure 4-1 shows that in the absence of Ca^{2+} (Fig. 4-A) or in the presence of 100 μM Ca^{2+} (Fig. 4-B) calreticulin was almost completely degraded by trypsin within 2 minutes of incubation. This indicates that at low concentrations of Ca^{2+} calreticulin was highly susceptible to digestion by trypsin. In contrast, at Ca^{2+} concentrations of 500 μM (Fig. 4-C) or 1 mM (Fig. 4-D) calreticulin exhibited resistance to trypsin digestion, and when digestion occurred it resulted in the generation of a 27-kDa protein which was highly resistant to further trypsin digestion (Fig. 4-1C and 4-1D). At high Ca^{2+} concentration the proteolytic activity of trypsin is highly increased (Bergmeyer, 1965; Burrell, 1993) yet the 27-kDa fragment was extremely resistant to trypsin digestion. Indeed, in the presence of 1 mM Ca^{2+}, the 27-kDa fragment was resistant to trypsin digestion for a period greater than 6 hours at 37°C (Fig. 4-1E). Similar results were obtained when calreticulin was digested with elastase, V8 protease and cathepsin G (Fig. 4-2).

Figure 4-1. Trypsin digestion of calreticulin.

Purified calreticulin was incubated with trypsin at 1:100 (trypsin/protein, w/w) at 37°C and in the presence or absence of a varying concentration of Ca^{2+}; no Ca^{2+} **(A)**, 100 μM Ca^{2+} **(B)**, 500 μM Ca^{2+} **(C)**, 1 mM Ca^{2+} **(D & E)** as indicated in the Figure. Aliquots were taken at time points indicated and the proteins were separated by SDS-PAGE and stained with Coomassie blue as described under "Methods", Chapter Two. The *arrow* indicates the 27-kDa protease resistant fragment of calreticulin. The position of molecular weight marker proteins (in kDa) is indicated. The molecular weight markers indicated are as follows: phosphoylase b (97,400), bovine serum albumin (66,200), ovalbumin (42,700), bovine carbonic anhydrase (31,000), and lysozyme (14,400).

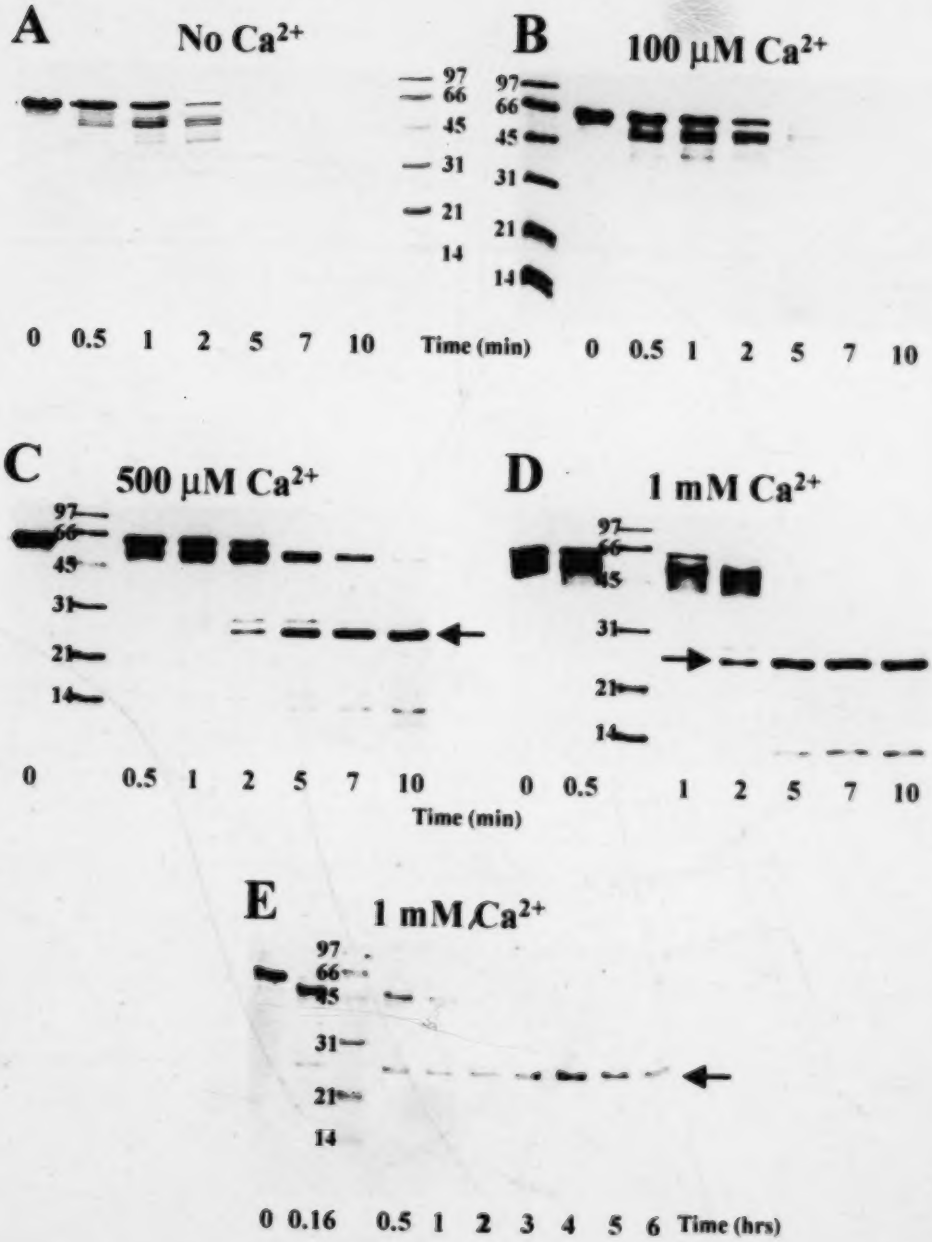
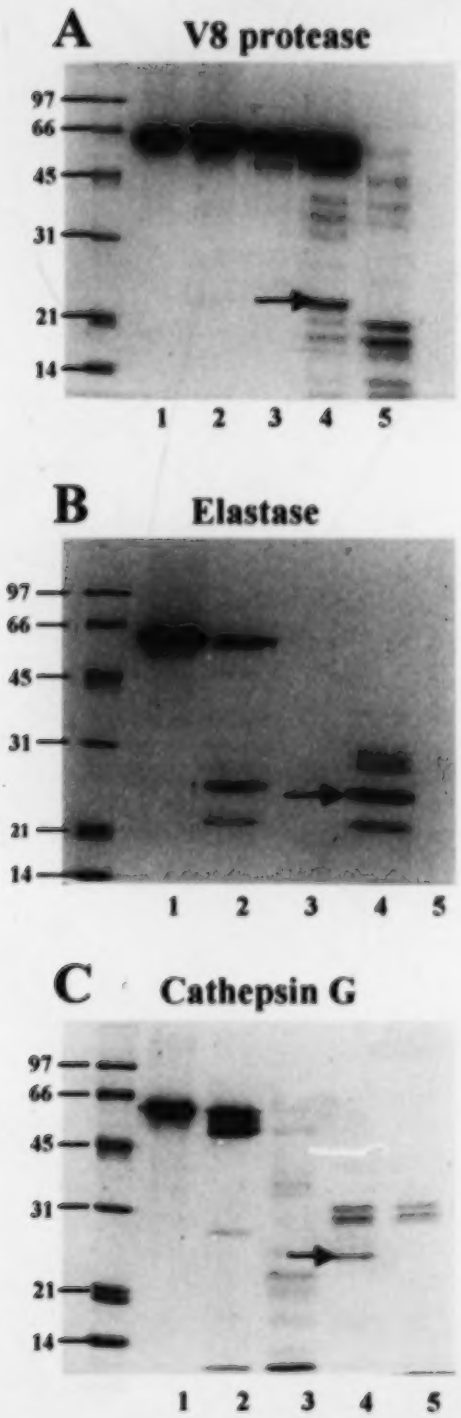

A
No Ca2+
B
100 µM Ca2+
97
66
45
31
21
14
0 0.5 1 2 5 7 10
Time (min)
C
500 µM Ca2+
D
1 mM Ca2+
E
1 mM Ca2+
0 0.16 0.5 1 2 3 4 5 6 Time (hrs)

Figure 4-2. Protease digestion of calreticulin.

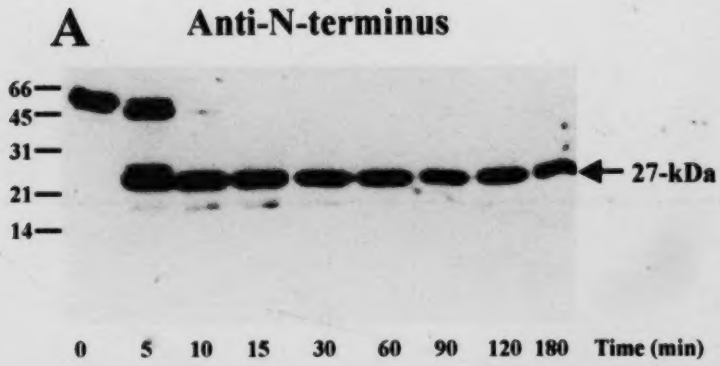
Purified calreticulin (*lane 1*) was digested with V8 protease (**A**), elastase (**B**), and cathepsin G (**C**) as described under "Methods", Chapter Two. Digestion was carried out in the presence of 5 mM EDTA or in the presence of 2 mM Ca^{2+}. *Lane 2*; digestion in the presence of 2 mM Ca^{2+} at calreticulin:enzyme = 320:1 (w/w); *lane 3*, digestion in the presence of 5 mM EDTA at calreticulin:enzyme = 320:1 (w/w); *lane 4*, digestion in the presence of 2 mM Ca^{2+} at calreticulin:enzyme = 20:1 (w/w); *lane 5*, digestion in the presence of 5 mM EDTA at calreticulin:enzyme = 20:1 (w/w). The *arrow* indicates the 27-kDa protease resistant fragment of calreticulin. The position of molecular weight marker proteins (in kDa) is indicated. Data provided by Steve Johnson.

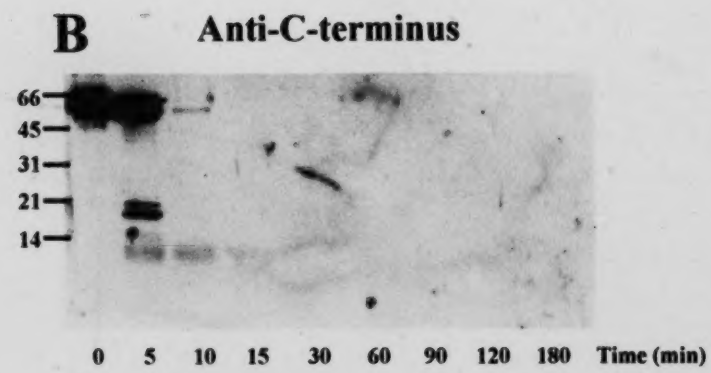

A
V8 protease
97
66
45
31
21
14
1 2 3 4 5
B
Elastase
97
66
45
31
21
14
1 2 3 4 5
C
Cathepsin G
97
66
45
31
21
14
1 2 3 4 5

To identify the Ca^{2+}-sensitive, trypsin-resistant 27-kDa proteolytic fragment of calreticulin, we carried out immunological analysis and NH_2-terminal amino acid sequence analysis. Calreticulin was digested with trypsin in the presence of 1 mM Ca^{2+} followed by Western blot analysis. First, we used a rabbit anti-synthetic peptide antibody, which recognizes the first 12 amino acids of mature calreticulin (Stendahl *et al.*, 1994; Van Delden *et al.*, 1992). Figure 4-3A shows that these antibodies, reacted with full-length calreticulin, as expected, and with the 27-kDa tryptic fragment of the protein. This indicates that proteolysis proceeded from the C-terminal region of the protein, leaving the amino terminal region intact. Next, we used a goat anti-calreticulin antibody, which recognizes the C-domain of calreticulin (Baksh and Michalak, 1991). As expected, this antibody recognized the full-length protein (Fig. 4-3B). However, the 27-kDa fragment was not recognized by this antibody (Fig. 4-3B), indicating that it did not include the C-domain of the protein. A short, C-domain fragment of calreticulin (~17-kDa) was detected by the goat antibody during the first 5 minutes of tryptic digestion, but this fragment was completely digested after 10 minutes of incubation with trypsin (Fig. 4-3B). The identity of the trypsin-resistant 27-kDa fragment was further confirmed by NH_2-terminal amino acid sequence analysis. The amino acid sequence we obtained (NH_2-E-A-Y-V-E-F-^{1}E-P-V-V-Y-F-K-E-Q-F-) corresponded to the NH_2-terminal amino acid sequence of full-length recombinant calreticulin expressed in *Pichia*. Identical NH_2-terminal amino acid sequence (NH_2-E-A-Y-V-E-F-^{1}E-P-V-V-Y-F-K-E-Q-F-) was also obtained for a 27-kDa fragment generated with elastase, V8 protease or cathepsin (Fig. 4-2) indicating that it was identical to the 27-kDa fragment generated with trypsin .

Figure 4-3. Immunological identification of protease resistant fragment of calreticulin.

Purified calreticulin was digested with trypsin (1:100; trypsin/protein; w/w) in the presence of 1 mM Ca^{2+} for the time indicated in the Figure. Proteins were separated by SDS-PAGE, transferred to nitrocelluose membrane and probed with antibodies against the N-terminus of calreticulin (**A**) or the C-terminus of the protein (**B**). The *arrow* indicates the 27-kDa protease resistant fragment. The position of molecular weight marker proteins (in kDa) is indicated.


A
Anti-N-terminus
66
45
31
21
14
27-kDa
0 5 10 15 30 60 90 120 180 Time (min)


B
Anti-C-terminus
66
45
31
21
14
0 5 10 15 30 60 90 120 180 Time (min)

These results indicate that the binding of Ca^{2+} alters calreticulins susceptibility to tryptic digestion, particularly in the NH_2-terminal region. This most likely results from Ca^{2+}-dependent conformational changes in the protein. It appears that at Ca^{2+} concentrations approximating those in full ER Ca^{2+} stores, the N-domain and some of the P-domain of calreticulin form a 27-kDa, protease resistant core (Fig. 4-3). Under these conditions the C-domain of the protein is in an extended conformation, hence accessible to digestion with the protease [1].

Zn^{2+}-dependent conformational changes in calreticulin

Zn^{2+} plays an important regulatory role in intracellular signal transduction and it is an important structural and functional element of many proteins including calreticulin (Vallee and Falchuk, 1993). Zn^{2+} binds to the N-domain of calreticulin, affecting its interaction with other proteins and inducing conformational changes, as determined by CD spectra analysis (Chapter Three, (Khanna *et al.*, 1986; Corbett *et al.*, 1999). Therefore, we examined the effect of Zn^{2+} on calreticulins protease resistance. Calreticulin was digested in the presence of 200 μM Zn^{2+}, a concentration that also

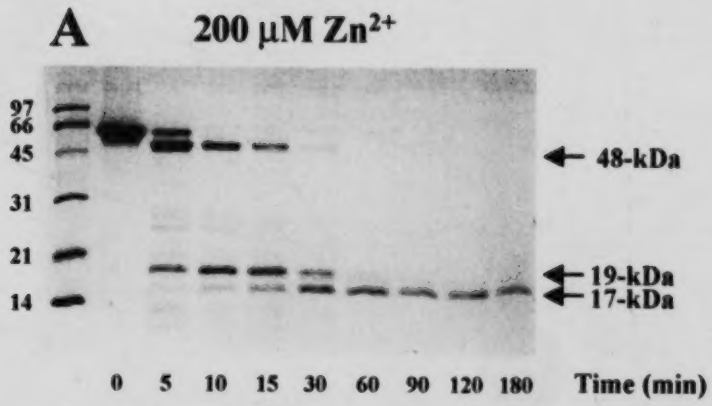
[1] Irfan Ashan, personal communication. Since the molecular weight (MW) of this fragment was determined directly from the SDS-PAGE gel, the values are likely to be somewhat over-estimated. The identity of this fragment was further confirmed by MALDI mass spectroscopy analysis after purification of the fragment by FPLC on a Resource Q column. Results revealed a molecular weight of 22.27-kDa. Since trypsin cleaves the C-terminal side of Lys or Arg residues it was possible to calculate that the COOH-terminal amino acid sequence was D-D-W-D-F-L-P-P-K-K-I-K^{192}-COO^-. This indicates that this protease resistant fragment consists of the entire N-domain of the protein and a part of the P-domain (22 amino acids). However, we shall continue to refer to this as the 27-kDa fragment, its apparent MW.

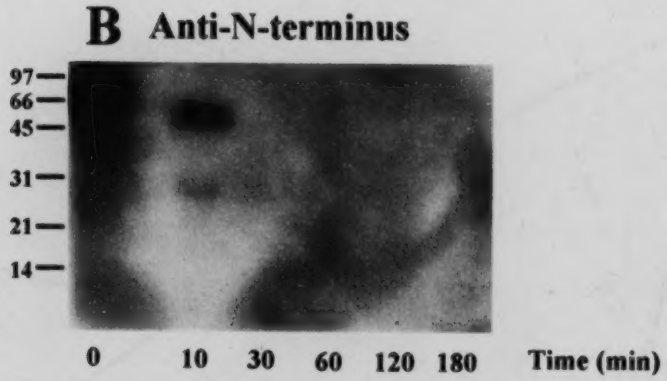
produces the largest conformational change in the protein (Khanna *et al.*, 1986; Corbett *et al.*, 1999).

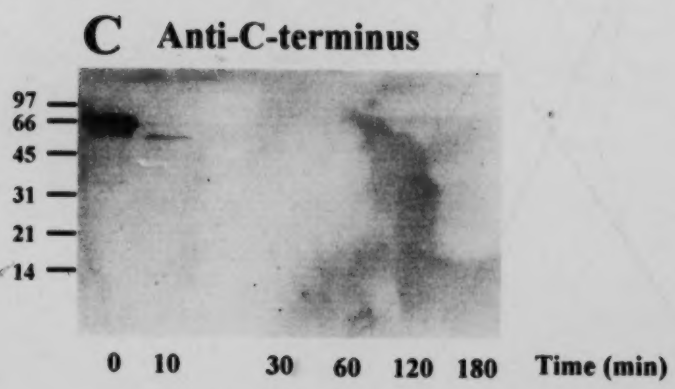
Figure 4-4A shows, that during the first 10 minutes of trypsin digestion, in the presence of 200 μM Zn^{2+}, two major proteolytic fragments were generated. One was a 48-kDa fragment that reacted with anti-N-terminal calreticulin antibodies and the other was a 19-kDa fragment that did not react (Fig. 4-4B). During prolonged digestion the 48-kDa fragment disappeared and the 19-kDa fragment was digested further, with the removal of an additional 40-50 NH_2-terminal residues, producing a 17-kDa trypsin-resistant fragment (Fig. 4-4A, 90-180 min). Neither the 19-kDa nor the 17-kDa fragment reacted with antibodies against the N- and C-terminal regions of calreticulin, suggesting that they represented an internal region of the protein (Fig. 4-4B & 4-4C). Amino acid sequence analysis of each fragment revealed two NH_2-terminal sequences, indicating that they are actually doublets. The N-terminal amino acid sequences of the 19-kDa fragments were NH_2-^{71}G-Q-P-L- and NH_2-^{78}T-V-K-H-E-Q-N-I-. The N-terminal amino acid sequences of the 17-kDa fragments were NH_2-^{118}D-S-x-Y-x-I-M- and NH_2-^{158}I-V-R-P-. The single disulfide bond in the sequence of calreticulin is found within the 19-kDa fragments, however is not present in the 17-kDa fragments. Breakage of the disulfide bond could be the rate-limiting factor in the production of the 17-kDa fragment from the 19-kDa fragment, which underlies the importance of the disulfide bond in maintaining structural integrity. Zn^{2+} has been reported to have either no effect on trypsin activity (Kirchgessner *et al.*, 1980) or to increase its proteolytic activity (Bergmeyer, 1965; Burrell, 1993) yet a 17-kDa fragment was relatively resistant to trypsin digestion in the presence of 200 μM Zn^{2+}.

Figure 4-4. (A-C) Zn^{2+}-dependent proteolysis of calreticulin.

Purified calreticulin was incubated with trypsin (1:100; trypsin/protein; w/w) at 37°C in the presence of 200 μM Zn^{2+} **(A-C)** or in the presence of 1 mM Ca^{2+} and 200 μM Zn^{2+} **(D-F)**. Digestion was stopped at time points indicated and the proteins were separated by SDS-PAGE, followed by staining with Coomassie blue **(A & D)** or probed with antibodies against the N-terminus of calreticulin **(B & E)** or the C-terminus of the protein **(C & F)** as described under "Methods", Chapter Two. The *arrows* indicate the position of 48-kDa, 19-kDa and 17-kDa protease resistant fragments of calreticulin **(A-C)** or the position of the 27-kDa, 26-kDa, 22-kDa and 19-kDa protease fragments of calreticulin **(D-F)**. The position of molecular weight marker proteins (in kDa) is indicated.


A
200 μM Zn2+
97
66
45
31
21
14
48-kDa
19-kDa
17-kDa
0 5 10 15 30 60 90 120 180
Time (min)


B Anti-N-terminus
97
66
45
31
21
14
0 10 30 60 120 180
Time (min)


C Anti-C-terminus
97
66
45
31
21
14
0 10 30 60 120 180
Time (min)

Previous studies have shown that Zn^{2+} binding to calreticulin induces conformational changes in the protein (Chapter Three, (Khanna *et al.*, 1986; Corbett *et al.*, 1999). Here we show that these changes lead to the formation of a central, protease-resistant core encompassing most of the P-domain of calreticulin. At the same time, the N-terminus and C-terminus of calreticulin may be in an extended conformation, which would explain their accessibility to digestion by the protease (Fig. 4-4).

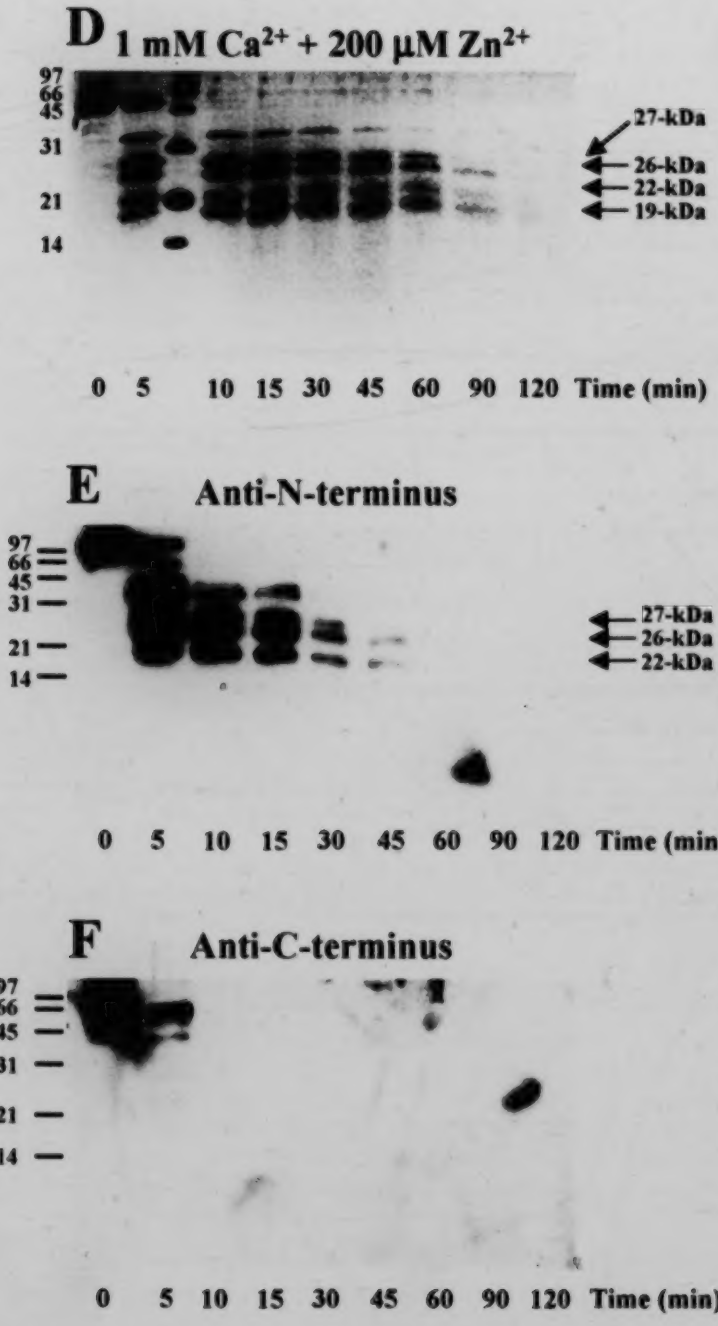
Trypsin digestion of calreticulin in the presence of both 1 mM Ca^{2+} and 200 μM Zn^{2+} resulted in the generation of proteolytic fragments almost identical to those observed in the presence of 1 mM Ca^{2+} and 200 μM Zn^{2+} alone. The exception was that in addition to the generation of the 27-kDa and 19-kDa fragments, there were two other proteolytic fragments generated; a 26-kDa and a 22-kDa, while the 17-kDa fragment was not generated (Fig. 4-4D). The 27-kDa, 26-kDa and 22-kDa fragments reacted with anti-N-terminal antibodies, while the 19-kDa fragment did not react (Fig. 4-4E) as was the case for the 19-kDa fragment produced in the presence of Zn^{2+} alone. As expected, none of the fragments reacted with antibodies against the C-terminal regions of calreticulin (Fig. 4-4F), indicating that proteolysis was proceeding from the C-terminal regions of the protein.

ATP-dependent conformational changes in calreticulin

Protein folding in the lumen of the ER requires ATP (Braakman *et al.*, 1992; Dorner and Kaufman, 1994; Dorner *et al.*, 1990). In order to investigate possible roles for ATP in the function of calreticulin, we carried out limited proteolytic digestion of calreticulin in the presence and absence of Mg^{2+}-ATP. Mg^{2+} and ATP have no effect on

Figure 4-4. (D-F) Zn^{2+}-dependent proteolysis of calreticulin.

Purified calreticulin was incubated with trypsin (1:100; trypsin/protein; w/w) at 37°C in the presence of 200 μM Zn^{2+} (A-C) or in the presence of 1 mM Ca^{2+} and 200 μM Zn^{2+} (D-F). Digestion was stopped at time points indicated and the proteins were separated by SDS-PAGE, followed by staining with Coomassie blue (A & D) or probed with antibodies against the N-terminus of calreticulin (B & E) or the C-terminus of the protein (C & F) as described under "Methods", Chapter Two. The *arrows* indicate the position of 48-kDa, 19-kDa and 17-kDa protease resistant fragments of calreticulin (A-C) or the position of the 27-kDa, 26-kDa, 22-kDa and 19-kDa protease fragments of calreticulin (D-F). The position of molecular weight marker proteins (in kDa) is indicated.

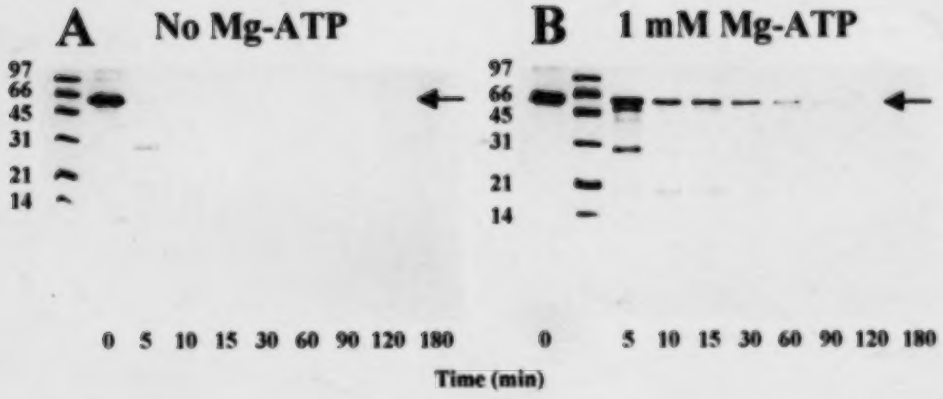

D
1 mM Ca2+ + 200 µM Zn2+
97
66
45
31
21
14
27-kDa
26-kDa
22-kDa
19-kDa
0 5 10 15 30 45 60 90 120 Time (min)
E
Anti-N-terminus
97
66
45
31
21
14
27-kDa
26-kDa
22-kDa
0 5 10 15 30 45 60 90 120 Time (min)
F
Anti-C-terminus
97
66
45
31
21
14
0 5 10 15 30 45 60 90 120 Time (min)

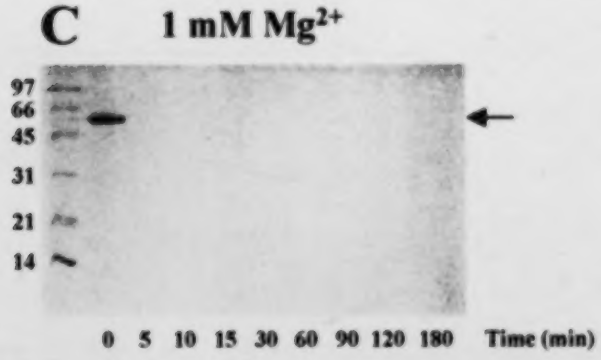
trypsin proteolytic activity (Burrell, 1993; Furukawa *et al.*, 1993). Figure 4-5B shows that in the presence of 1 mM Mg^{2+}-ATP full-length calreticulin was quite resistant to trypsin digestion, and was protected for the first 45 minutes of incubation. The identity of the "protected" full-length calreticulin was confirmed by NH_2-terminal amino acid sequence analysis. The amino acid sequence we obtained (NH_2-E-A-Y-V-E-F-^{1}E-P-V-V-Y-F-K-E-Q-F-) corresponded to the NH_2-terminal amino acid sequence of full-length recombinant calreticulin expressed in *Pichia*. Mg^{2+} alone had no protective effect (Fig. 4-5C). ADP and AMP also did not have any protective effect (Fig. 4-5D & E), nor did GTP or GDP (data not shown) indicating that they did not bind to calreticulin. After 5 minutes of incubation with trypsin, in addition to the full-length protein, a 30-kDa major proteolytic fragment was observed (Fig. 4-5B). The 30-kDa fragment was rapidly digested to small peptides. It appears that calreticulin binds Mg^{2+}-ATP and that this significantly reduces its susceptibility to proteolytic digestion, likely *via* a conformational change. A striking difference in the protective effects of Ca^{2+}, Zn^{2+}, and ATP against tryptic digestion of calreticulin is that only ATP protected the C-domain of the protein.

Next, to mimic a more physiological environment, we investigated the combined effect of Ca^{2+}, Zn^{2+}, and Mg^{2+}-ATP on calreticulins sensitivity to trypsin. Figure 4-6 shows the effect of ATP on the conformation of calreticulin, in the presence of 200 μM Zn^{2+} (**A**) or 1 mM Ca^{2+} (**B**). In the presence of Zn^{2+} and Mg^{2+}-ATP, the digestion of calreticulin by trypsin produced a 55-kDa trypsin-resistant fragment (Fig. 4-6A). Under these conditions the full-length calreticulin also showed significant resistance (more than 2 hours) to proteolysis. The NH_2-terminal amino acid sequence of the 55-kDa fragment was NH_2-^{78}T-V-K-H-E-Q-N-I-D-, which is identical to the NH_2-terminal amino acid

Figure 4-5. ATP-dependent proteolysis of calreticulin.

Purified calreticulin was incubated with trypsin (1:100; trypsin/protein; w/w) at 37°C in the presence or absence of 1 mM Mg^{2+}-ATP (**B & A** respectively), the presence of 1 mM Mg2+ (**C**), 1 mM ADP (**D**), and 1 mM AMP (**E**). Digestion was stopped at time points indicated and the proteins were separated by SDS-PAGE and stained with Coomassie blue as described under "Methods", Chapter Two. The *arrows* indicate the position of full-length calreticulin (60-kDa). The position of molecular weight marker proteins (in kDa) is indicated.


A
No Mg-ATP
B
1 mM Mg-ATP
97
66
45
31
21
14
0 5 10 15 30 60 90 120 180
0 5 10 15 30 60 90 120 180
Time (min)


C
1 mM Mg2+
97
66
45
31
21
14
0 5 10 15 30 60 90 120 180
Time (min)

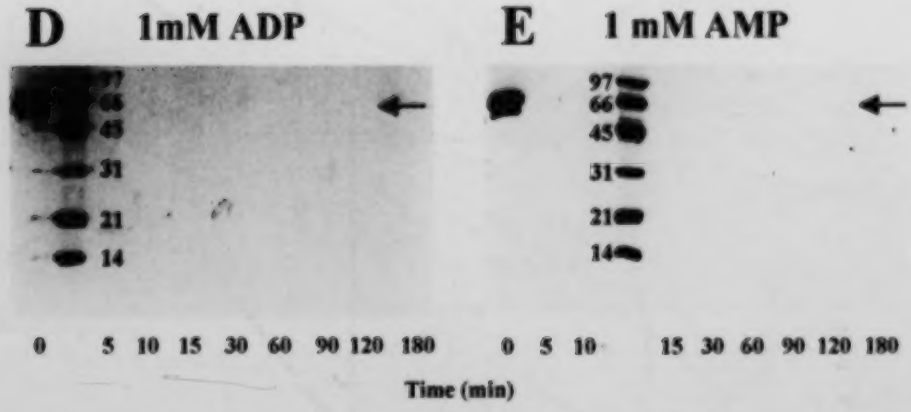
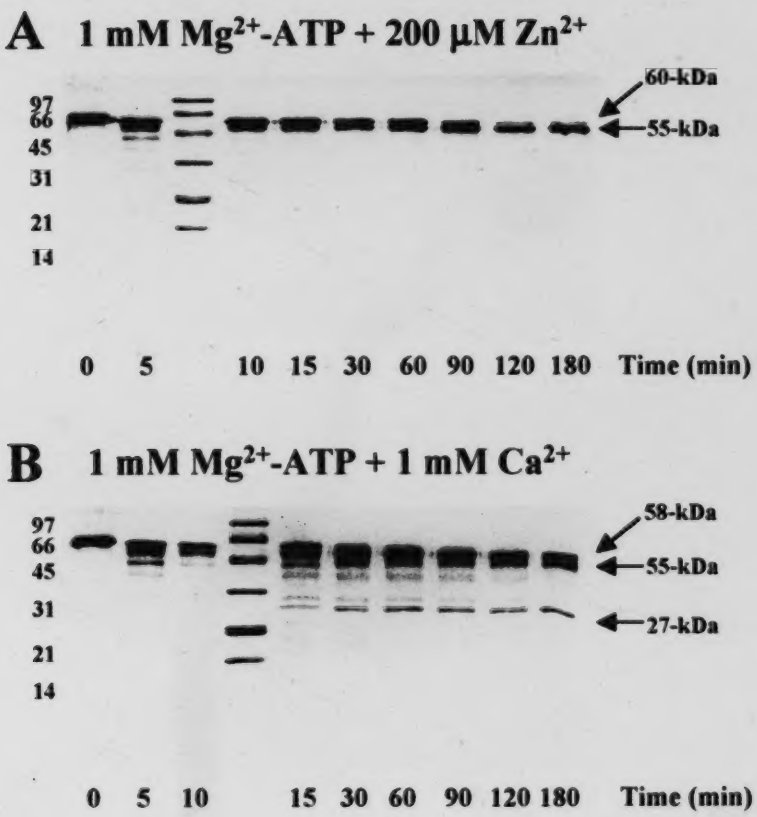

D
1mM ADP
E
1 mM AMP
31
21
14
97
66
45
31
21
14
0 5 10 15 30 60 90 120 180
0 5 10 15 30 60 90 120 180
Time (min)

Figure 4-6. Combined effect of ATP, Zn^{2+} and Ca^{2+} on the proteolysis of calreticulin.

Purified calreticulin was incubated with trypsin (1:100; trypsin/protein, w/w) at 37°C in the presence of 1 mM Mg^{2+}-ATP and 200 μM Zn^{2+} **(A)** or in the presence of 1 mM Mg^{2+}-ATP and 1 mM Ca^{2+} **(B)**. The proteins were separated by SDS-PAGE and stained with Coomassie blue. The *arrows* indicate the position of full-length calreticulin (60-kDa), 58-kDa, 55-kDa and 27-kDa protease resistant fragments of the protein. The position of molecular weight marker proteins (in kDa) is indicated.


A 1 mM Mg2+-ATP + 200 µM Zn2+
97
66
45
31
21
14
60-kDa
55-kDa
0 5 10 15 30 60 90 120 180 Time (min)
B 1 mM Mg2+-ATP + 1 mM Ca2+
97
66
45
31
21
14
58-kDa
55-kDa
27-kDa
0 5 10 15 30 60 90 120 180 Time (min)

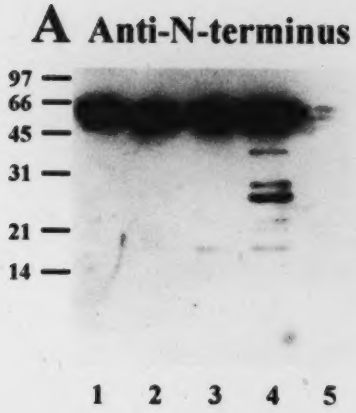
sequence of the 19-kDa tryptic fragment produced in the presence of Zn^{2+} alone (Fig. 4-4A). The difference between these two fragments was that the 19-kDa fragment was missing the C-domain of calreticulin. This supports our previous observation that Mg^{2+}-ATP protects the C-domain of calreticulin from tryptic digestion.

Figure 4-6B shows that tryptic digestion of calreticulin in the presence of Mg^{2+}-ATP and Ca^{2+} resulted in the generation of three major fragments; one 58-kDa, one 55-kDa and one 27-kDa. The NH_2-terminal amino acid sequence of the 58-kDa fragment was NH_2-E-A-Y-V-E-F-^{1}E-P-V-V-Y-F-K- and it corresponded to a full-length recombinant calreticulin. This indicated that under these conditions proteolysis of calreticulin proceeded from the C-terminal region of the protein. The NH_2-terminal amino acid sequence of the 27-kDa fragment was also NH_2-E-A-Y-V-E-F-^{1}E-P-V-V-Y-F-K-E-Q-F-. This sequence, which corresponds to the amino terminal sequence of full-length, mature calreticulin, is identical to the NH_2-terminal amino acid sequence of the 27-kDa trypsin-resistant fragment obtained in the presence of 500 μM Ca^{2+} (Fig. 4-1C & D, 4-2 and 4-3A).

To further identify these proteolytic fragments, calreticulin was digested with trypsin for 15 minutes in the presence of Mg^{2+}-ATP, Mg^{2+}-ATP and Zn^{2+}, or Mg^{2+}-ATP and Ca^{2+}. The digestion was followed by SDS-PAGE and Western blot analysis with anti-N-terminus (Fig. 4-7A) and anti-C-terminus (Fig. 4-7B) antibodies. As expected, in the absence of ions and nucleotides the 15 minutes tryptic digestion of calreticulin resulted in its complete proteolysis (Fig. 4-7A and D, *lane 5*). However, after 15 minutes tryptic digestion in the presence of Mg^{2+}-ATP, Mg^{2+}-ATP and Zn^{2+}, or Mg^{2+}-ATP and Ca^{2+}, some full-length calreticulin remained undigested and was recognized by both the

Figure 4-7. Immunological identification of protease resistant fragment of calreticulin.

Purified calreticulin (*lane 1*) was digested with trypsin (1:100; trypsin/protein; w/w) for 15 min. in the presence of 1 mM Mg^{2+}-ATP (*lane2*); 1 mM Mg^{2+}-ATP and 200 μM Zn^{2+} (*lane 2*), 1 mM Mg^{2+}-ATP and 1 mM Ca^{2+} (*lane 3*); and alone (*lane 4*). Proteins were separated by SDS-PAGE, transferred to nitrocelluose membrane and probed with antibodies against the N-terminus of calreticulin (**A**) or the C-terminus of the protein (**B**). The position of molecular weight marker proteins (in kDa) is indicated.


A Anti-N-terminus
97
66
45
31
21
14
1 2 3 4 5
B Anti-C-terminus
66
45
31
21
14
1 2 3 4 5

anti-N-terminus (Fig. 4-7A) and anti-C-terminus (Fig. 4-7B) antibodies. The 27-kDa tryptic fragment obtained in the presence of Mg^{2+}-ATP and Ca^{2+}, as expected, interacted with the anti-N-terminal antibody (Fig. 4-7A, *lane 4*) but not with the anti-C-terminal antibody (Fig. 4-7B, *lane 4*). A schematic representing all of the protease resistant fragments of purified calreticulin after digestion with trypsin is shown in Appendix 1 & 2.

Biophysical analysis of ATP-dependent conformational changes in calreticulin

To examine ATP-dependent conformational changes in calreticulin, far UV CD analysis of the protein was carried out in the absence and presence of Mg^{2+}-ATP, Mg^{2+}-ATP + Zn^{2+}, Mg^{2+}-ATP + Ca^{2+}, and Mg^{2+}-ATP + Ca^{2+} + Zn^{2+}. Control experiments were carried out in the absence and presence of 1 mM Ca^{2+}, 1 mM Zn^{2+} and 1 mM Mg^{2+}. The CD spectra were analyzed for secondary structural elements by the Contin ridge regression analysis program (Provencher and Glöckner, 1981), and representative data are shown. (Table 4-1, Fig. 4-8). The apoprotein has ~10% α-helix and 66% β-sheet-β-turn, similar to values published earlier (Corbett *et al.*, 1999). Figure 4-8 shows the CD spectra of calreticulin in the presence of Ca^{2+} (Fig.4-4A), Zn^{2+} (Fig. 4-4B) and Mg^{2+}-ATP (Fig. 4-4C). Calreticulin showed no change upon addition of Ca^{2+} (Fig. 4-4A), but underwent a reduction in α-helix and an increase in combined β-sheet-β-turn upon addition of Zn^{2+} (Fig. 4-8B), as reported earlier (Chapter Three, (Khanna *et al.*, 1986). The addition of Mg^{2+}-ATP alone (Fig. 4-8C), and of Mg^{2+}-ATP with Zn^{2+} and/or Ca^{2+}, resulted in small (just outwith experimental error) changes in secondary structural elements. Since CD changes are averaged over the entire molecule, they are often not a very sensitive measure of small conformational changes.

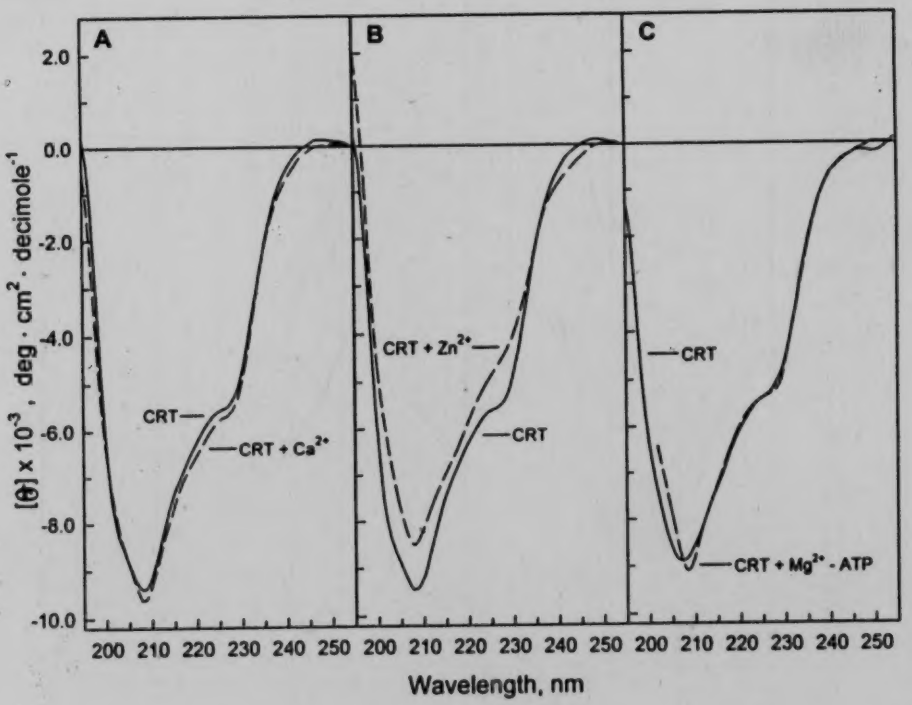
Table 4-1: Provencher-Glöckner secondary structural analysis of calreticulin in the absence and presence of Mg^{2+}-ATP and metal

Sample	α-Helix	β-Sheet	β-Turn	Remainder	Scale factor
Calreticulin	0.10 ± 0.005	0.37 ± 0.010	0.29 ± 0.012	0.23 ± 0.008	0.077
Calreticulin + 1 mM Ca^{2+}	0.09 ± 0.005	0.34 ± 0.008	0.29 ± 0.009	0.26 ± 0.008	1.046
Calreticulin + 1 mM Zn^{2+}	0.12 ± 0.003	0.41 ± 0.005	0.22 ± 0.006	0.25 ± 0.003	1.006
Calreticulin + 1 mM Mg^{2+}	0.16 ± 0.003	0.33 ± 0.006	0.24 ± 0.006	0.27 ± 0.004	1.001
Calreticulin + 1 mM Mg^{2+}-ATP	0.13 ± 0.007	0.30 ± 0.013	0.29 ± 0.015	0.29 ± 0.012	1.074
Calreticulin + 1 mM Mg^{2+}-ATP + 1 mM Ca^{2+}	0.15 ± 0.008	0.25 ± 0.013	0.26 ± 0.016	0.33 ± 0.013	1.018
Calreticulin + 1 mM Mg^{2+}-ATP + 1 mM Zn^{2+}	0.16 ± 0.006	0.36 ± 0.011	0.20 ± 0.013	0.29 ± 0.009	1.000
Calreticulin + 1 mM Mg^{2+}-ATP + 1 mM Ca^{2+} + 1 mM Zn^{2+}	0.19 ± 0.007	0.28 ± 0.011	0.23 ± 0.014	0.30 ± 0.011	1.049

K. Oikawa & R. Luty performed the CD analysis.

Figure 4-8. CD analysis of calreticulin

CD spectra of purified calreticulin was carried out as described under "Methods", Chapter Two. The data are plotted as molar ellipiticity versus wavelength for calreticulin in the absence (———) and presence of Ca^{2+} (A), Zn^{2+} (B), and Mg^{2+}-ATP (C) (- - - - -). Kim Oikawa performed the CD analysis.


A
B
C
2.0
0.0
-2.0
-4.0
-6.0
-8.0
-10.0
[θ] x 10^-3, deg · cm^2 · decimole^-1
CRT
CRT + Ca2+
CRT + Zn2+
CRT
CRT
CRT + Mg2+ - ATP
200 210 220 230 240 250
200 210 220 230 240 250
200 210 220 230 240 250
Wavelength, nm

The binding of ATP to calnexin causes its ER luminal region to dimerize (Ou *et al.*, 1995). Therefore, we used sedimentation velocity analysis to explore possible effects of ATP on the oligomerization of calreticulin. Table 4-2 indicates that $S_{20,w}$ values of calreticulin, at a concentration of 3.07 mg/ml, are indistinguishable under all conditions examined. Apparently, calreticulin exists as a monomeric protein and the binding of ATP to calreticulin does not induce it to form oligomers.

Calreticulin binds ATP

ATP-dependent conformational changes in calreticulin, as measured by its altered sensitivity to proteolysis, suggest that the protein binds ATP. In order to demonstrate directly that calreticulin binds ATP, we carried out ATP-affinity chromatography of the purified protein (Fig. 4-9A). CL-2B-agarose chromatography of the protein was carried out as a control (Fig. 4-9B). Calreticulin was applied to the column, followed by extensive washing with the binding buffer and then elution of ATP binding proteins with 5 mM ATP. Importantly, a significant amount of calreticulin was bound to the ATP-agarose and was specifically eluted with 5 mM ATP (Fig. 4-9A, *5 mM ATP*). In contrast, calreticulin did not bind to the CL-2B-agarose control column (Fig. 4-9B). Subsequent application of 2 M NaCl eluted no further calreticulin, indicating that calreticulin had been specifically eluted from the column by ATP (Fig. 4-9A, *lane 2M NaCl*). These experiments demonstrate that calreticulin binds ATP.

Chaperones localized in the lumen of the ER are also known to hydrolyze ATP (Flynn *et al.*, 1989; Guthapfel *et al.*, 1996; Munro and Pelham, 1986; Wei *et al.*, 1995; Wei and Hendershot, 1995). Therefore, we tested calreticulins ability to hydrolyze ATP,

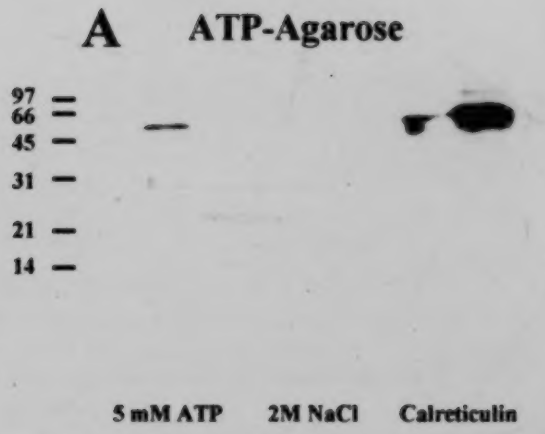
Table 4-2: Sedimentation velocity of calreticulin in the absence and presence of ATP and metals

Sample	Concentration *mg/ml*	$S_{observed}$	$S_{20,w}$
Calreticulin (CRT)	3.07	2.8977	3.0495
CRT + 1 mM Mg^{2+}-ATP	3.07	2.8755	3.0258
CRT + 1 mM Mg^{2+}-ATP + 1 mM Ca^{2+}	3.07	2.9526	3.1067
CRT + 1 mM Mg^{2+}-ATP + 1mM Zn^{2+}	3.07	2.9333	3.0862

L. Hicks performed the sedimentation velocity experiments.

Figure 4-9. Direct binding of ATP to calreticulin.

Calreticulin was incubated at 37°C for with *ATP-Agarose* or *Control Agarose* (CL-2B Agarose) beads in a binding buffer as described under "Methods", Chapter Two. ATP bound proteins were eluted with a binding buffer containing 5 mM Mg^{2+}-ATP (*lane 5 mM ATP*) followed by a wash with 2 M NaCl (*lane 2 M NaCl*). Eluted proteins were separated on SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were probed with anti-C-terminus calreticulin antibodies as described under "Methods", Chapter Two. The position of molecular weight marker proteins (in kDa) is indicated.


A
ATP-Agarose
97
66
45
31
21
14
5 mM ATP
2M NaCl
Calreticulin
B
Control Agarose
97
66
45
31
21
14
5 mM ATP
2M NaCl
Calreticulin

in the presence and absence of 100 μM and 1 mM Ca^{2+}, using a coupled enzyme system (Michalak *et al.*, 1984). Microsomal vesicles isolated from HEK293 cells were used as a control (Table 4-3) (Michalak *et al.*, 1984). We found that calreticulin does not hydrolyze ATP under these conditions (Table 4-3).

PDI and ERp57 induce conformational changes in calreticulin

ER luminal chaperones form structural and functional complexes (Booth and Koch, 1989; Nigam *et al.*, 1994; Baksh *et al.*, 1995a; Corbett *et al.*, 1999; Oliver *et al.*, 1999). For example, calreticulin interacts with ERp57 (Chapter Three, (Corbett *et al.*, 1999; Molinari and Helenius, 1999; Oliver *et al.*, 1999) and in a Ca^{2+}-dependent manner with PDI (Baksh *et al.*, 1995a; Corbett *et al.*, 1999). We have shown that these interactions induce conformational changes in both ERp57 and PDI (Fig. 3-7 (Corbett *et al.*, 1999). We therefore wanted to examine what conformational changes are induced in calreticulin due to binding of either ERp57 or PDI. Sensitivity of calreticulin to proteolysis was therefore tested in the presence of ERp57 and PDI to examine a role of these specific protein-protein interactions in conformational changes of calreticulin. Calreticulin was digested with trypsin in the presence of PDI and in the absence of Ca^{2+}, the condition required to promote interaction between these two proteins (Baksh *et al.*, 1995a; Corbett *et al.*, 1999). Protein mixtures were separated by SDS-PAGE, blotted to nitrocellulose membrane and probed with antibodies raised against the N- and the C-terminus of calreticulin. Figure 4-10A and 4-10B shows calreticulin alone (in the absence of PDI, ERp57 and Ca^{2+}), as expected, was rapidly digested by trypsin and could not be detected with anti-calreticulin antibodies (Fig. 4-10A and 4-10B). In contrast when

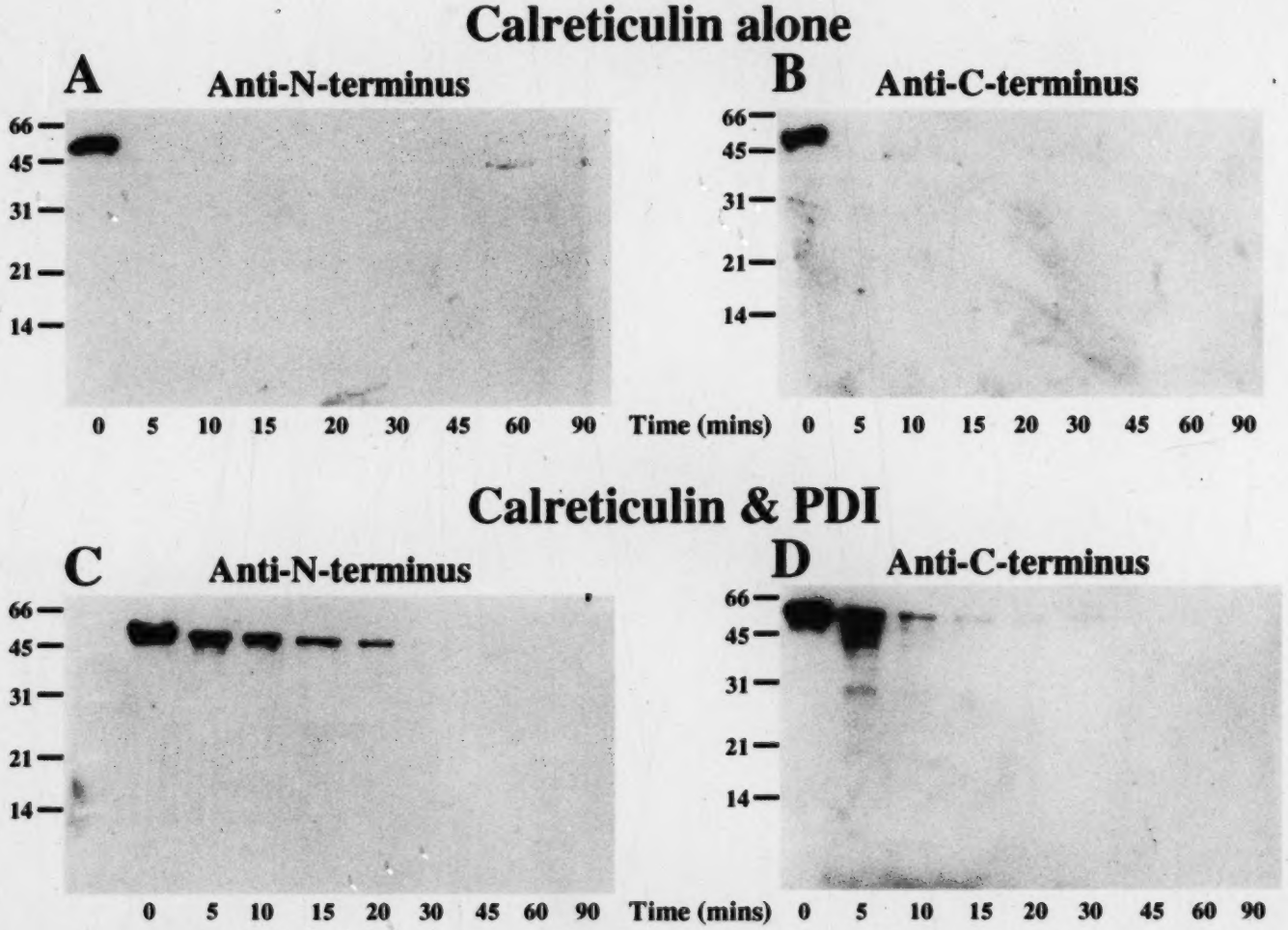
Table 4-3: ATP hydrolysis by calreticulin

ATPase activity of calreticulin and microsomal vesicles isolated from HEK293 cells was measured as described under "Experimental Procedures". Data are the ± S.E. of three independent experiments. ND, not detectable. Data provided by Dr. Christi Andrin.

Sample	ATPase activity *nmol/Pi/min/mg protein*
Calreticulin	<0.001
Calreticulin + 100 μM Ca^{2+}	ND
Calreticulin + 1 mM Ca^{2+}	ND
HEK293 microsomes + 1 mM Ca^{2+}	14.3 ± 2.1

Figure 4-10: Proteolysis of calreticulin in the presence of PDI

Purified calreticulin was incubated with trypsin (1:100; trypsin/protein; w/w) at 37°C alone (**A & B**), in the presence of PDI (**C & D**). Digestion was stopped at time points indicated and the proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane and probed with antibodies against the N-terminus of calreticulin (**A & C**) or the C-terminus of the protein (**B & D**) as described under "Methods", Chapter Two. The position of molecular weight marker proteins (in kDa) is indicated.

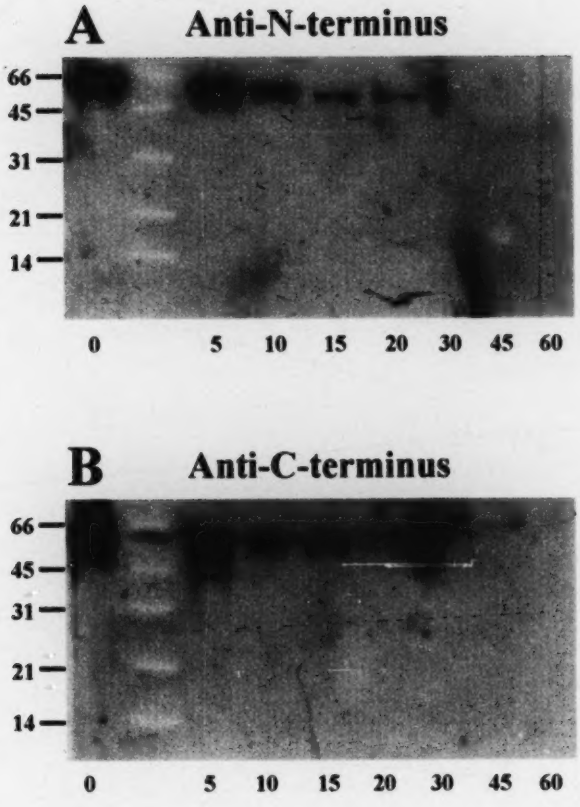

Calreticulin alone
A
Anti-N-terminus
B
Anti-C-terminus
66
45
31
21
14
0 5 10 15 20 30 45 60 90
Time (mins)
0 5 10 15 20 30 45 60 90
Calreticulin & PDI
C
Anti-N-terminus
D
Anti-C-terminus
66
45
31
21
14
0 5 10 15 20 30 45 60 90
Time (mins)
0 5 10 15 20 30 45 60 90

calreticulin formed complexes with PDI, the protein was relatively resistant to trypsin digestion (Fig. 4-10C and 4-10D). As tested with anti-N-terminal calreticulin antibodies, under these conditions the protein was resistant to proteolysis for over 15 minutes (Fig. 4-10C) indicating that PDI binding to calreticulin induced conformational changes in the protein. The most C-terminal region of calreticulin although more protected, was undetectable by western with antibodies against the C-terminus of calreticulin after ~ 5 minutes of digestion (Fig. 4-10D).

Interaction with ERp57 induces conformational changes in calreticulin or results in masking of trypsin sites that also results to the protein being more resistant to trypsin digestion (Fig. 4-11A and 4-11B). As with PDI, interaction of calreticulin with ERp57 resulted in protection of the most N-terminal regions of calreticulin that were detectable with antibodies against the N-terminus of the protein. In addition, however interaction of calreticulin with ERp57 also resulted in the most C-terminal region of the protein being protected. After 15 minutes of digestion both the N- and C-terminus of calreticulin could be detected with antibody (Fig. 4-11A and 4-11B). We have previously documented that the N-domain of calreticulin is involved in interactions with both PDI and ERp57 (Chapter Three, (Corbett *et al.*, 1999). It now appears that interaction of ERp57 with calreticulin, can induce conformational changes in the most C-terminus of calreticulin as seen by its trypsin digestion protection. These results indicate that interactions with other ER luminal chaperones can significantly affect the conformation and stability of calreticulin. Hence, chaperones may not only interact to facilitate in protein folding, but these interactions may also provide stability to the chaperone network.

Figure 4-11: Proteolysis of calreticulin in the presence of ERp57.

Purified calreticulin was incubated with trypsin (1:100; trypsin/protein; w/w) at 37°C in the presence of ERp57 (**A & B**). Digestion was stopped at time points indicated and the proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane and probed with antibodies against the N-terminus of calreticulin (**A**) or the C-terminus of the protein (**B**) as described under "Methods", Chapter Two. The position of molecular weight marker proteins (in kDa) is indicated.


A
Anti-N-terminus
66
45
31
21
14
0
5
10
15
20
30
45
60
B
Anti-C-terminus
66
45
31
21
14
0
5
10
15
20
30
45
60

Discussion

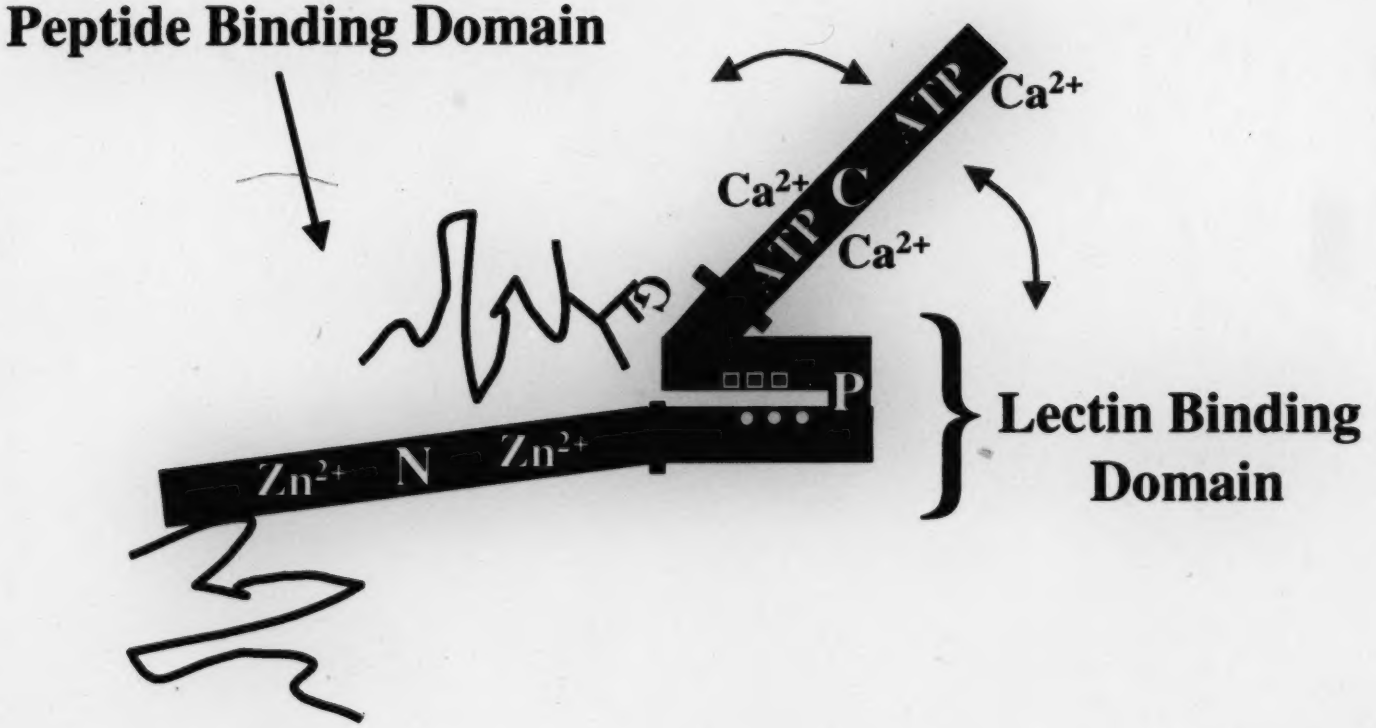
In this study, we show that environmental conditions in the lumen of the ER can affect calreticulin's conformation as documented by its sensitivity to proteolytic digestion with trypsin, elastase, V8 protease, and cathepsin G. Specifically the binding of Ca^{2+}, Zn^{2+}, and Mg^{2+}-ATP to calreticulin appears to induce conformational changes in the protein, leading to the formation of a protease-resistant core structure. The protease-resistant core always encompasses the P-domain of the protein. Ca^{2+} binding protects the N-terminal (N-domain and some of the P-domain) region of calreticulin, Zn^{2+} protects its P-domain and Mg^{2+}-ATP protects the full-length protein. These results suggest that the N- and C-domains of calreticulin are flexible and may undergo frequent, ion-induced changes in conformation. These changes in conformation may explain how Ca^{2+} and Zn^{2+} alter the function of calreticulin. We also demonstrate that interaction of both PDI and ERp57 with calreticulin result in conformational changes in the protein, and these may be important for the chaperone function of the protein. The interaction of PDI and ERp57 with calreticulin protects calreticulin's N-domain, while interaction with ERp57 also protects the C-domain of calreticulin. That calreticulin be flexible in solution is entirely consistent with its biological role as a molecular chaperone and the underlying requirement for the protein to associate effectively but transiently with a large number of structurally different glycoproteins. In this study, we also show that calreticulin binds ATP. Despite this, the protein does not hydrolyze ATP and ATP does not induce formation of calreticulin oligomers. We conclude that changes in the concentration of Ca^{2+}, Zn^{2+} and Mg^{2+}-ATP, in the lumen of the ER, and interaction with other ER luminal chaperones significantly affect the conformation, stability, and function, of calreticulin.

Earlier structural characterization of calreticulin predicted that the general shape of calreticulin would consist of a large globular N-terminal region followed by a more elongated C-terminal region (Fliegel *et al.*, 1989a). An extension of these results was recently provided by Bouvier and Stafford (Bouvier and Stafford, 2000). They show that calreticulin exists as an asymmetric elongated molecule in solution, where certain regions of the protein are likely to adopt extended conformations in solution. In agreement with our results they suggest that calreticulin is likely to be intrinsically flexible in solution with the ability to undergo local conformational changes upon ligand binding (Bouvier and Stafford, 2000). Results from this group also indicate that the P-domain of calreticulin is likely involved in minimal inter-domain interactions (Bouvier and Stafford, 2000). Until recently there was no information on the three-dimensional structure of calreticulin. Studies on the NMR spectroscopy structure of calreticulins P-domain (amino acids 189-288) are in direct agreement with the results of Bouvier and Stafford (Ellgaard *et al.*, 2001a; Ellgaard *et al.*, 2001b). These studies indicate that the N- and C-termani of the P-domain of calreticulin are in close proximity with one another, and suggest that in the native protein the P-domain constitutes a finger-like protrusion from the calreticulin core structure (Ellgaard *et al.*, 2001a; Ellgaard *et al.*, 2001b). A schematic model for the structure of calreticulin encompassing this data in addition to my results is proposed in Figure 4-12.

ATP is required to support correct folding, and correct disulfide bond formation in many proteins, and it is an important component of the ER luminal environment. Here we provide the first direct evidence that calreticulin binds ATP, although we found no

Figure 4-12: Schematic model of the structure of calreticulin

Calreticulin is divided into three structural and functional domains, N-, P- and C-domain. The N-domain binds Zn^{2+} and is also involved in interactions with non-glucosylated folding proteins. The central P-domain binds mono-glucosylated nascent proteins and also binds Ca^{2+} with high affinity and low capacity. This domain is extended from the calreticulin core structure and is involved in minimal inter-domain interactions. The C-domain binds Ca^{2+} with low affinity and high capacity and is likely involved in binding ATP. This domain appears flexible in solution, and is likely to undergo ion- and nucleotide-induced changes in conformation (as indicated by the orange arrows).

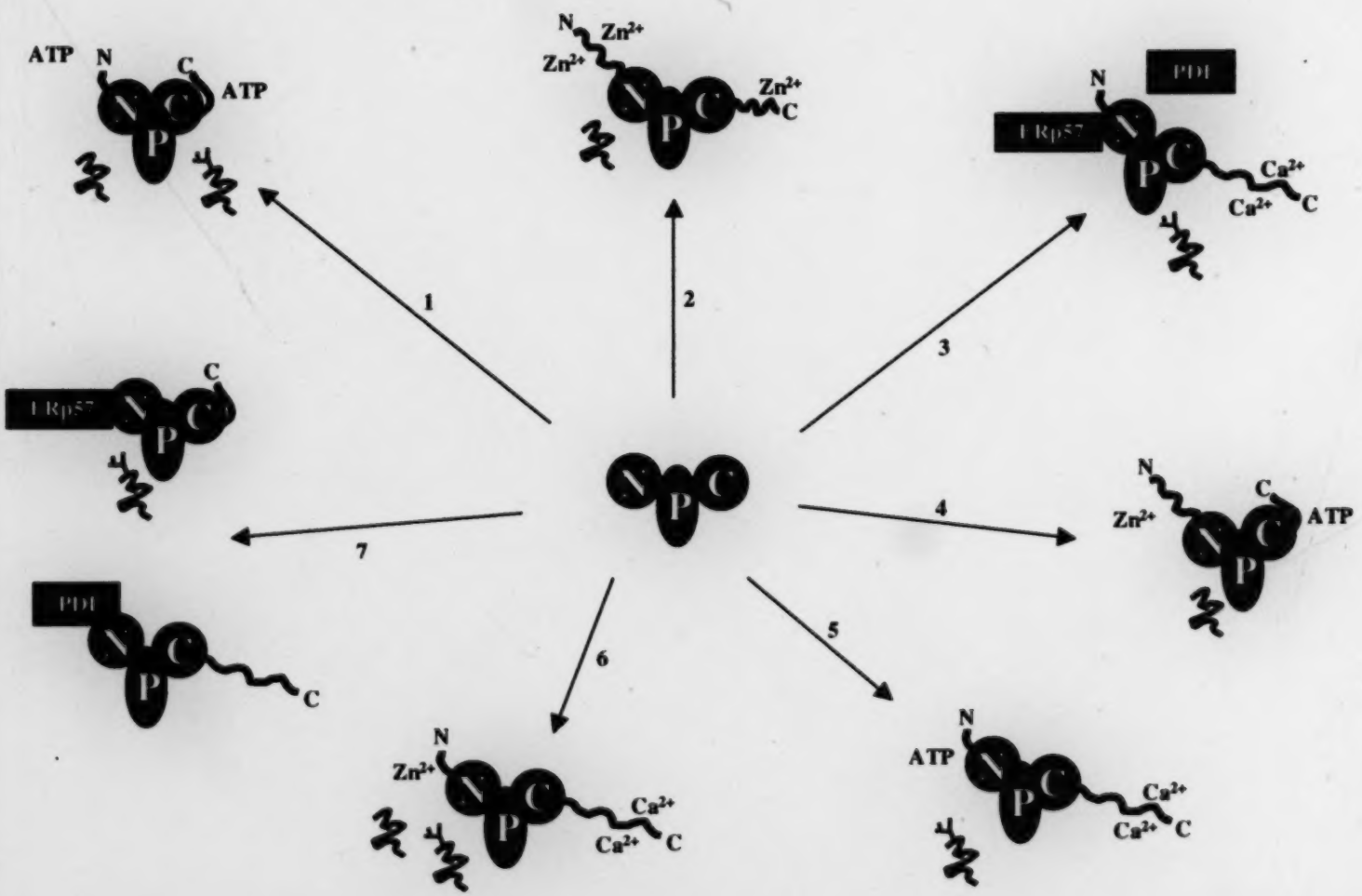

Peptide Binding Domain
Ca2+
ATP
C
Ca2+
ATP
Ca2+
P
Zn2+
N
Zn2+
Lectin Binding Domain

evidence that it hydrolyzes the ATP. We also show that ATP significantly reduces calreticulins susceptibility to tryptic-digestion, indicating that ATP stabilizes specific conformation of the protein. Importantly, calreticulin does not bind ADP and AMP as determined by its sensitivity to proteolysis.

Figure 4-13 shows a proposed model for conformational changes of calreticulin in the ER lumen. The most distinctive feature of ATP binding to calreticulin is that it protected the full-length protein from tryptic digestion (Fig. 4-13 **(1)**). This is in contrast to Ca^{2+} and Zn^{2+}. For example, Ca^{2+} binding to the low affinity and high capacity C-domain results in conformational changes in C-terminal region of calreticulin making it susceptible to proteolysis (Fig. 4-13 **(3)**). In the presence of Zn^{2+}, the N- and C-domain regions of calreticulin also appear to take an extended conformation that is susceptible to proteolysis (Fig. 4-13 **(2)**). However, Zn^{2+} has less effect on the structure of calreticulin in the presence of Ca^{2+} (Fig. 4-13 **(6)**), indicating that Ca^{2+}-binding may induce structural changes that overcome Zn^{2+}-induced conformational changes. While there is no obvious nucleotide-binding site in calreticulin, the protection of the C-domain by ATP is suggestive that its binding involves the C-domain. This is further supported by the ATP-dependent protection of the C-domain in the presence of Zn^{2+} (Fig. 4-13 **(4)**). Using ANS, a probe frequently used to monitor the surface hydrophobicity of proteins, Saito *et al* (Saito *et al.*, 1999) demonstrated that both ATP and Zn^{2+} increased the surface hydrophobicity of calreticulin. However, the nature of the environment reported by ANS with either ATP or Zn^{2+} appears to be different. Binding of ATP and Zn^{2+} was accompanied by distinctly different conformational changes (Saito *et al.*, 1999), consistent with the notion that ATP and Zn^{2+} expose hydrophobic polypeptide binding

Figure 4-13. A hypothetical model of protease sensitivity of calreticulin.

(1): -effect of ATP on the proteolysis of calreticulin. In the presence of ATP full-length calreticulin is resistant to digestion with trypsin. Ca^{2+} binding to the C-domain of calreticulin in the presence of ATP (5) results in conformational changes in the protein leading to unmasking of the C-domain of the protein. (4): shows that in the presence of ATP and Zn^{2+} the N-domain of the protein is an extended conformation. (2) Zn^{2+} binding to the N-domain of calreticulin results in a conformational change leading to unmasking of the N- and C-domains of the protein and to the formation of a protease resistant core consisting of the P-domain and a part of the N-domain of the protein. Ca^{2+} binding to the C-domain of calreticulin (3) induces conformational change resulting in proteolysis of the C-domain and formation of a protease resistant core encompassing the N- and P-domains of the protein. (6): this shows that the binding of Zn^{2+} and Ca^{2+} induce conformational changes resulting in proteolysis of the C-domain and some of the N-domain. (7): shows that interaction of PDI and ERp57 with the N-domain of calreticulin results in its protection, while ERp57 interaction also protects its C-domain.


ATP
N
C
ATP
P
N
Zn2+
Zn2+
Zn2+
C
P
N
PDI
ERp57
P
C
Ca2+
Ca2+
C
1
2
3
ERp57
C
P
N
C
Zn2+
ATP
P
4
7
PDI
P
C
5
6
N
Zn2+
P
Ca2+
Ca2+
C
N
ATP
P
Ca2+
Ca2+
C

sites in different regions of the calreticulin molecule. This further supports our thoughts that while Zn^{2+} is binding the N-domain, ATP is inducing conformational changes in the C-domain. Furthermore, Cala (Cala, 1999) has demonstrated a tight association of a phosphate-containing compound within the C-domain of calreticulin. They localized binding to a single calreticulin peptide – ^{367}LKEEEEDKK. As this peptide contains no phosphorylatable residues, they suggest that calreticulin may bind a phosphate or phosphate-containing molecule at this site (Cala, 1999). ATP has less effect on the C-domain of calreticulin in the presence of Ca^{2+} (Fig. 4-13 **(5)**) indicating that Ca^{2+} may compete with ATP for binding to the C-domain. ATP binding could play a role in protection of this region of the protein, or in regulation of its activity. Furthermore, ATP causes a slight enhancement in oligosaccharide binding to the P-domain of calreticulin, whereas Ca^{2+} enhances this binding significantly (Vassilakos *et al.*, 1998). Ca^{2+}- and ATP-dependent conformational changes of calreticulin described here may play important role in the regulation of this chaperone function of calreticulin. Results from Saito *et al.* (Saito *et al.*, 1999) demonstrate that ATP affects interaction between calreticulin and misfolded proteins. ATP enhanced calreticulins ability to suppress aggregation of both glycosylated and non-glycosylated substrates (Saito *et al.*, 1999). *Via* these interactions, the chaperone activity of calreticulin may be modified, or controlled, by changes in the concentration of Ca^{2+} and ATP in the lumen of the ER. Other recent observations support this suggestion. Similarly, ATP-induced conformational changes in BiP, another ER chaperone, are necessary for it to release bound peptide (Wei *et al.*, 1995; Wei and Hendershot, 1995). ATP binding to calreticulin results in dramatic changes in the structure of the protein (Fig. 4-13 **(1)**) and it may play a role in the

regulation of calreticulin chaperone activity by increasing its surface hydrophobicity and enhancing its ability to suppress aggregation of both glycosylated and non-glycosylated unfolded proteins.

Mg^{2+}-ATP binds to calnexin, induces protein oligomerization and increases calnexin's sensitivity to proteolysis (Ou *et al.*, 1995). In contrast, we found that ATP binding to calreticulin does not promote its oligomerization, however, it protects calreticulin from proteolysis. These findings indicate that, while ATP may play an important role in both proteins, its effects are significantly different. In agreement with our results Bouvier *et al* (Bouvier and Stafford, 2000) demonstrated that calreticulin exists as a monomeric protein.

Calreticulin binds Zn^{2+} and Zn^{2+} is found in intracellular compartments known to contain calreticulin such as the ER, SR, synaptic vesicles and secretory granules (Frederickson *et al.*, 1987; Holm *et al.*, 1988; Reddy *et al.*, 1989; Picello *et al.*, 1992). This binding involves highly conserved histidine residues in the N-domain of the protein (Baksh *et al.*, 1995b) and it induces conformational changes as measured by CD spectroscopy and fluorescence techniques (Chapter Three, (Baksh *et al.*, 1995b; Corbett *et al.*, 1999; Saito *et al.*, 1999). Zn^{2+} also affects interactions between calreticulin and other ER chaperones and between calreticulin and misfolded proteins (Baksh *et al.*, 1995b; Corbett *et al.*, 1999; Saito *et al.*, 1999). In this study, we show that Zn^{2+}-dependent conformational changes in calreticulin result in the formation of a protease-resistant central core (Fig. 4-13 **(2)**). The isolation of a stable fragment encompassing most of the P-domain from a thermolysin digestion mixture has since been described, further confirming our results (Bouvier and Stafford, 2000). The N-domain and the C-

domain of the protein are unprotected and, therefore, may be in an extended conformation (Fig. 4-13 **(2)**). Saito *et al* (Saito *et al.*, 1999) reports that Zn^{2+} enhances calreticulins ability to reduce the aggregation of newly synthesized proteins (Saito *et al.*, 1999). The N- and C-domains of calreticulin may be involved in this, *via* interaction with misfolded proteins (not glycoproteins) (Fig. 4-13 **(2)**). Indeed, CD spectra analysis shows that Zn^{2+} binding increases the hydrophobicity of calreticulin, which could enhance its interaction with hydrophobic regions of misfolded proteins.

Although much Ca^{2+} in the lumen of the ER is stored by binding to chaperones, a significant portion of the Ca^{2+} is free (Meldolesi and Pozzan, 1998a). In response to external stimuli, free $[Ca^{2+}]_{ER}$ fluctuates (Miyawaki *et al.*, 1997; Meldolesi and Pozzan, 1998a). The free $[Ca^{2+}]_{ER}$ changes from as high as 500 μM to as low as 1 μM (Miyawaki *et al.*, 1997). In this study, we have shown, for the first time, that Ca^{2+} binding also alters calreticulins conformation as monitored by its susceptibility to tryptic digestion. At low Ca^{2+} concentrations (>100 μM, empty Ca^{2+} stores), Ca^{2+} binds only to high affinity sites located in the P-domain of calreticulin (Baksh and Michalak, 1991). Under these conditions, calreticulin appears to exist in a conformation that is "relaxed" (exposed and highly susceptible to proteolysis). When the concentration of Ca^{2+} is increased to >500 μM (corresponding to full Ca^{2+} stores) Ca^{2+} also binds to the C-domain of calreticulin. When this happens the protein appears to undergo a conformational change, forming a core that is protease-resistant and which encompasses the highly conserved N-domain and the lectin-like P-domain of calreticulin while the C-domain is in an extended conformation able to bind Ca^{2+} (Fig. 4-13 **(3)**). Most importantly, it is low affinity Ca^{2+} binding in the C-domain that is responsible for these changes, and not high affinity

binding in the P-domain. These results are consistent with the C-terminus of calreticulin being most exposed and conformationally flexible in solution and therefore more susceptible to proteolysis by trypsin. In agreement with these results temperature-dependent proteolysis with thermolysin revealed that the N- and P-domains of calreticulin form a stable core region, while the highly acidic C-terminus of the protein however, is more susceptible to digestion (Bouvier and Stafford, 2000). It is likely that these Ca^{2+} induced conformational changes in calreticulin are responsible for the Ca^{2+}-dependent interaction of calreticulin with PDI and ERp57 (Fig. 4-13 (3)) (Chapter Three, (Khanna *et al.*, 1986; Corbett *et al.*, 1999). Studies on human placental calreticulin show that the C-domain is susceptible to proteolytic cleavage, while the N- and P-domains form a proteolytically stable tight association (Hojrup *et al.*, 2001). Furthermore, Ca^{2+} binding to the ER luminal region of calnexin induces conformational changes in calnexin as measured by its resistance to proteinase K digestion (Ou *et al.*, 1995). Chaperone function of both, calreticulin and ER luminal region of calnexin, may be regulated by Ca^{2+}-dependent changes in their conformation. Our results also indicate that the $[Ca^{2+}]_{ER}$ can significantly affect the conformation and stability of calreticulin. For example, the degradation of both PDI and calreticulin by ERp72 is Ca^{2+}-dependent (Urade *et al.*, 1993). Specifically, the degradation of PDI is enhanced at 1 mM Ca^{2+}, whereas the degradation of calreticulin is inhibited (Urade *et al.*, 1993).

We have previously demonstrated that calreticulin interacts with ERp57, and with PDI in a Ca^{2+}-dependent manner (Baksh *et al.*, 1995a; Corbett *et al.*, 1999). Interaction between calreticulin and PDI is restricted to the N-domain of calreticulin, while the Ca^{2+}-dependent dissociation of this complex is confined to the C-domain of the protein. Here

we have demonstrated that interaction of PDI with the N-domain of calreticulin results in its protection, while the C-domain of calreticulin is likely extended from this protein complex, and is susceptible to trypsin digestion (Fig. 4-13 **(7)**). ERp57 also forms complexes with calreticulin, binding of calreticulin to ERp57 is initiated by a Ca^{2+}-independent conformational change in ERp57 (Fig. 4-13 **(7)**) followed by a Ca^{2+}-dependent modulation of the complex due to Ca^{2+} binding the C-domain of calreticulin (Fig. 4-13 **(3)**) (Chapter Three, (Corbett *et al.*, 1999). Interaction of ERp57 also occurs through calreticulins N-domain and subsequently results in its protection from trypsin digestion. In contrast to PDI, however, interaction of ERp57 with calreticulin also protects its C-domain and therefore is unlikely to be extended from the protein complex (Fig. 4-13 **(7)**).

The formation of the Ca^{2+}-dependent protease resistant 27-kDa fragment of calreticulin may have important physiological and pathophysiological implications. For example, autoantibodies against calreticulin are present in the sera of a number of autoimmune conditions (Eggleton *et al.*, 1997). The NH_2-terminal region of calreticulin, corresponding to the Ca^{2+} resistant 27-kDa fragment, contains most of the antigenic sites (Eggleton *et al.*, 2000). The same protease resistant fragment of calreticulin is produced in the VERO76 cells infected with a wild type Rubella virus [2]. It is tempting to speculate that viral infection, or other cellular stresses, may induce release of calreticulin and expose the protein to proteolytic enzymes in the presence of high Ca^{2+} concentrations. This would result in the production of high levels of the protease resistant calreticulin fragment possessing a highly antigenic conformation.

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[2] H. Nakhasi, personal communication

Similar NH_2-terminal fragments of calreticulin has been isolated as secreted proteins from an Epstein-Barr virus-immortalized cell line, and consists of the entire N-domain of the protein. This peptide was named vasostatin and shown to inhibit angiogenesis and endothelial cell proliferation and suppress tumor growth (Pike *et al.*, 1998; Pike *et al.*, 1999). One possible mechanism for the generation of this fragment is that calreticulin is proteolytically processed by ER luminal proteases, under conditions of full ER Ca^{2+} stores, giving rise to the 27-kDa protease resistant fragment or vasostatin, which would then be secreted from the cells. As shown in Figure 4-2B and 4-2C digestion of calreticulin with elastase and cathepsin G, results in cleavage of the C-terminus of the protein, leaving the stable 27-kDa protease resistant fragment. Elastase and cathepsin G are two of the major proteases released from leucocytes during inflammation (Eggleton *et al.*, 2000). Under the high [Ca^{2+}] seen extracellularly, cleavage by elastase or cathepsin G would result in cleavage of the C-terminus of the protein, leaving the stable N-terminal fragment. In support of this it is known that during apoptosis ER vesicles containing calreticulin bleb off from the surface of dying cells (Eggleton *et al.*, 1997). Release of calreticulin from apoptotic blebs or from cells undergoing necrosis at the site of inflammation, would expose calreticulin to proteolytic enzymes, therefore making it susceptible to proteolytic attack by leucocyte secretory enzymes such as cathepsin H and elastase. Either way, this could provide a mechanism for the release of calreticulin into the circulation, where it inhibits angiogenesis, and suppresses tumor growth (Pike *et al.*, 1998; Pike *et al.*, 1999). Similarly, this may be the mechanism by which calreticulin, the autoantigen, is released. As an angiogenesis inhibitor that specifically targets proliferating endothelial cells, vasostatin has a unique

potential for the treatment of cancer and other diseases. More recent work has demonstrated that vasostatin, in combination with interleukin-12 (IL-12), halted tumor growth of human Burkitt lymphoma, colon carcinoma, and ovarian carcinoma (Yao *et al.*, 2000). One concern, however, is that vasostatin, which consists of the N-domain of calreticulin, is also the most antigenic part of the molecule.

In this study conformational changes in the ER chaperone calreticulin have been defined in the presence of Ca^{2+}, Zn^{2+}, ATP, and the ER luminal chaperones PDI and ERp57. The fact that calreticulin is flexible in solution is consistent with its biological role as a molecular chaperone and the fact that it has to associate effectively but transiently with a large number of substrates *in vivo*. It will be important to determine precisely how such conformational changes regulate the physiological function of the protein intracellularly. Moreover, the existence of proteolytic fragments of calreticulin released from cells undergoing stress have been observed to interact with proteins and cells of the immune system, knowledge of these events will also help us understand the pathological significance of calreticulin released into the extracellular environment.

Chapter Five

Summary and General Discussion

Summary of Thesis

The following are the major observations of my thesis work:

1. I have studied interactions between calreticulin and two related ER membrane chaperones, PDI and ERp57, and investigated the role that Ca^{2+} plays in these protein-protein interactions. I found that calreticulin interacts with PDI and that this interaction is Ca^{2+}-dependent.

2. I have provided the first direct evidence that calreticulin interacts with ERp57, and although this interaction is not Ca^{2+}-dependent, Ca^{2+} binding to calreticulin can influence the interaction of these two chaperones. Formation of the ERp57-calreticulin complex was initiated by a Ca^{2+}-independent conformational change in ERp57 followed by additional Ca^{2+}-dependent conformational changes in the complex. Interaction of calreticulin with both chaperones occurs via interactions with the N-domain of the protein, while the Ca^{2+} sensitivity of the calreticulin-PDI and the Ca^{2+}-dependent modulation of the calreticulin-ERp57 complex was confined to the high capacity Ca^{2+} binding, C-domain of calreticulin.

3. I have shown that like numerous other ER lumenal chaperones that calreticulin is an ATP-binding protein but that it does not contain any detectable ATPase activity.

4. I investigated the effects of the ER lumenal environment on the conformation of calreticulin. Specifically I utilized the binding of Ca^{2+}, Zn^{2+} and ATP or interaction of PDI or ERp57 to provide details on the three-dimensional structure of calreticulin. I show that binding of Ca^{2+}, Zn^{2+} and ATP or interaction of calreticulin with PDI or ERp57 affects the tertiary structure of calreticulin, as indicated by changes in its sensitivity to protease digestion.

General Discussion

The work presented in my thesis demonstrates that the ER chaperones calreticulin and PDI interact with one another and do so in a Ca^{2+}-dependent fashion. Importantly, the Ca^{2+}-dependency varies over the range of free $[Ca^{2+}]_{ER}$ measured during re-filling and emptying of ER Ca^{2+} stores. Interaction between calreticulin and PDI is restricted to the NH_2-terminal region (N-domain) of calreticulin, but most importantly, Ca^{2+}-binding to the physiologically relevant high-capacity, low-affinity Ca^{2+}-binding site (C-domain) in calreticulin is responsible for control of these interactions. For example, calreticulin binds reversibly to PDI *in vitro* at the low free $[Ca^{2+}]_{ER}$ observed when Ca^{2+} stores are empty (<50 μM), and others have shown that this protein-protein interaction results in a reduction of PDI chaperone activity and decreased binding of Ca^{2+} to calreticulin (Baksh *et al.*, 1995a; Zapun *et al.*, 1998). By contrast, at the high free $[Ca^{2+}]_{ER}$ observed when Ca^{2+} stores are refilled (>500 μM), PDI does not interact with calreticulin. When PDI is not interacting with calreticulin it displays high chaperone activity (Baksh *et al.*, 1995a; Zapun *et al.*, 1998). In agonist stimulated cells during emptying of ER Ca^{2+} stores PDI and calreticulin would tightly interact, possibly inducing the release of unfolded proteins from PDI which would then be free to interact with other ER chaperones in the ER lumen. This may allow a flux of proteins to move from one chaperone to the next.

I also have provided the first direct evidence that ERp57 interacts with and forms protein complexes with calreticulin. As with PDI, the N-domain of calreticulin plays a key role in this protein-protein interaction and Ca^{2+} binding to the high capacity C-domain of calreticulin is involved in modulation of this chaperone complex. However, unlike the calreticulin-PDI complex, binding of calreticulin to ERp57 is initiated by a

Ca^{2+}-independent, conformational change in ERp57 followed by a Ca^{2+}-dependent modulation of the complex. So although the initial interaction between calreticulin and ERp57 is independent of $[Ca^{2+}]$, ERp57 is affected indirectly in two ways. Firstly, at high free $[Ca^{2+}]_{ER}$, Ca^{2+} binding to calreticulin induces conformational changes in ERp57, presumably allowing closer interaction of the protein with folding intermediates, thereby assisting disulfide bond formation in newly synthesized glycoproteins. In support of this, it has been shown that ERp57 disulfide isomerase activity is increased in the presence of either calreticulin or calnexin (Zapun *et al.*, 1998). Secondly, at low free $[Ca^{2+}]_{ER}$, calreticulin binds mono-glucosylated glycoproteins only weakly. This suggests that calreticulin-ERp57 chaperone complexes are probably not functional in Ca^{2+}-depleted ER, whereas they are likely to be functional when Ca^{2+} stores are full and glycoproteins are bound to calreticulin, calnexin or both. Interactions between calreticulin, PDI and ERp57 are restricted to the N-terminal region of the protein (N-domain), while the Ca^{2+} sensitivity is confined to the C-terminal part of the protein (C-domain) (Chapter Three) suggesting that the C-domain of calreticulin may play a role of Ca^{2+} "sensor" in the ER lumen.

Later work by Oliver *et al* (Oliver *et al.*, 1999) confirmed some of our results and demonstrated the specific interaction between *in vitro* synthesized calreticulin and ERp57 in the absence of other ER components. In addition to this, they established that ERp57 forms discrete complexes with calreticulin and calnexin *in vivo*, and in fact these interactions can be seen in Lec23 cells, which lack glucosidase I, the protein necessary to produce glycoprotein substrates capable of binding to calnexin and calreticulin (Oliver *et al.*, 1999). These results are in agreement with the results presented here indicating that

calreticulin and ERp57 do form a direct interaction with one another and can interact in the absence of glycoprotein substrate.

Previous work has shown that calreticulin can form structural and functional protein complexes with other chaperones. Nigam *et al* (Nigam *et al.*, 1994) isolated a set of ER resident proteins (calreticulin, BiP, GRP94, PDI, ERp72, p50 and a 46-kDa protein) that bound to a denatured protein affinity column in an ATP- and Ca^{2+}-dependent manner. Perhaps it was binding of Ca^{2+} to the low affinity, high capacity C-domain that was responsible for this dissociation. Association of calreticulin with GRP94, GRP78 and calnexin has also been reported (Tatu and Helenius, 1997). Complexes of ER lumenal chaperones are associated with maturation of thyroglobulin (Kim and Arvan, 1995; Kuznetsov *et al.*, 1997) and apolipoprotein B (Linnik and Herscovitz, 1998). The association and dissociation of these and other ER lumenal chaperone complexes may also be regulated by Ca^{2+} binding as described for interaction of calreticulin with PDI and ERp57 (Chapter Three).

The function of other chaperones in the lumen of the ER is sensitive to fluctuations in free $[Ca^{2+}]_{ER}$. For example, at low free $[Ca^{2+}]_{ER}$ the activity of BiP is inhibited and its association with proteins is stabilized (Kassenbrock and Kelly, 1989; Gaut and Hendershot, 1993a; Gaut and Hendershot, 1993b;). Ca^{2+}-binding can also regulate interactions between GRP94 and other proteins (Koyasu *et al.*, 1989). At low free $[Ca^{2+}]_{ER}$ protein synthesis, glycoprotein processing and transport competence are blocked (Ivessa *et al.*, 1995; Subramanian and Meyer, 1997). It appears that these effects might be mediated *via* Ca^{2+}-dependent changes in protein-protein interactions in the lumen of the ER. Overall, changes in the free $[Ca^{2+}]_{ER}$ might result in cycles of Ca^{2+}-

dependent association and dissociation between ER molecular chaperones (Figure 5-1). This is important, because it may facilitate passage of certain newly synthesized proteins from one chaperone to another during the folding process and allow the modulation of complexes of molecular chaperones. This would be in agreement with the earlier proposed 'calcium-matrix' hypothesis of Koch and Sambrook (Koch, 1987; Sambrook, 1990). This proposed that in agonist stimulated cells fluctuating Ca^{2+} concentrations would allow the maintenance of an immobile chaperone network with protein folding intermediates being concentrated in the mobile fluid phase thus enhancing their folding. It is also apparent from the work presented in this thesis that fluctuating Ca^{2+} concentrations allows for the dissociation or modulation of inter-chaperone interactions, thus supporting the 'calcium-matrix' hypothesis proposed over 12 years ago.

Moreover, Ca^{2+} in the ER lumen may also play a role in the stability of lumenal chaperones. For example, degradation of PDI and calreticulin by the protease activity of ERp72 is Ca^{2+}-dependent (Urade *et al.*, 1993). PDI degradation is enhanced in the presence of 1 mM Ca^{2+}, but degradation of calreticulin is inhibited by the presence of 1 mM Ca^{2+} (Urade *et al.*, 1993). At low $[Ca^{2+}]_{ER}$ association between PDI and calreticulin may protect PDI from degradation. When Ca^{2+} stores are full, PDI and calreticulin would dissociate possibly resulting in PDI being more susceptible to degradation by ERp72, while calreticulin would be protected.

In most cells Ca^{2+} has a major signaling function when it is released into the cytosol from intracellular stores. The release and re-uptake of Ca^{2+} results in a continuous fluctuation in the free $[Ca^{2+}]_{ER}$. It is now known that changes in the free $[Ca^{2+}]_{ER}$ affect many functions of the ER including the synthesis and secretion of proteins and

Figure 5-1. (A) Ca^{2+}-dependent dynamics in the lumen of the ER.

This diagram shows a model of events occurring in response to agonist activated Ca^{2+} fluctuations in the lumen of the ER. These events affect interactions between chaperones and the function of chaperones.

In A (1) during agonist activation Ca^{2+} is released from the ER *via* the $InsP_3$ receptor (IP_3R)/Ca^{2+} channel, resulting in decreased free $[Ca^{2+}]_{ER}$ (<50 μM). Under these conditions, there are only limited interactions between proteins in the lumen of the ER and unfolded polypeptides.

Broken arrows indicate that there is presently no direct evidence for interactions between calreticulin and SERCA2b and between calreticulin and $InsP_3$ receptor. Solid arrows indicate demonstrated pathways. The differing intensity of the background represents differences in Ca^{2+} concentration in depleted and refilled Ca^{2+} stores. CRT, calreticulin; CNX, calnexin; CD, central domain of calnexin; PDI, protein disulfide isomerase, Gluc II, glucosidase II; UDP-Gluc transferase, UDP-glucose:glycoprotein glucosyltransferase; G, glucose residue. Reprinted from *TiBS*, 25, Corbett, E.F., and Michalak, M. Calcium, a signaling molecule in the endoplasmic reticulum. 307-311.© 2000, with permission from *Elsevier Science*.

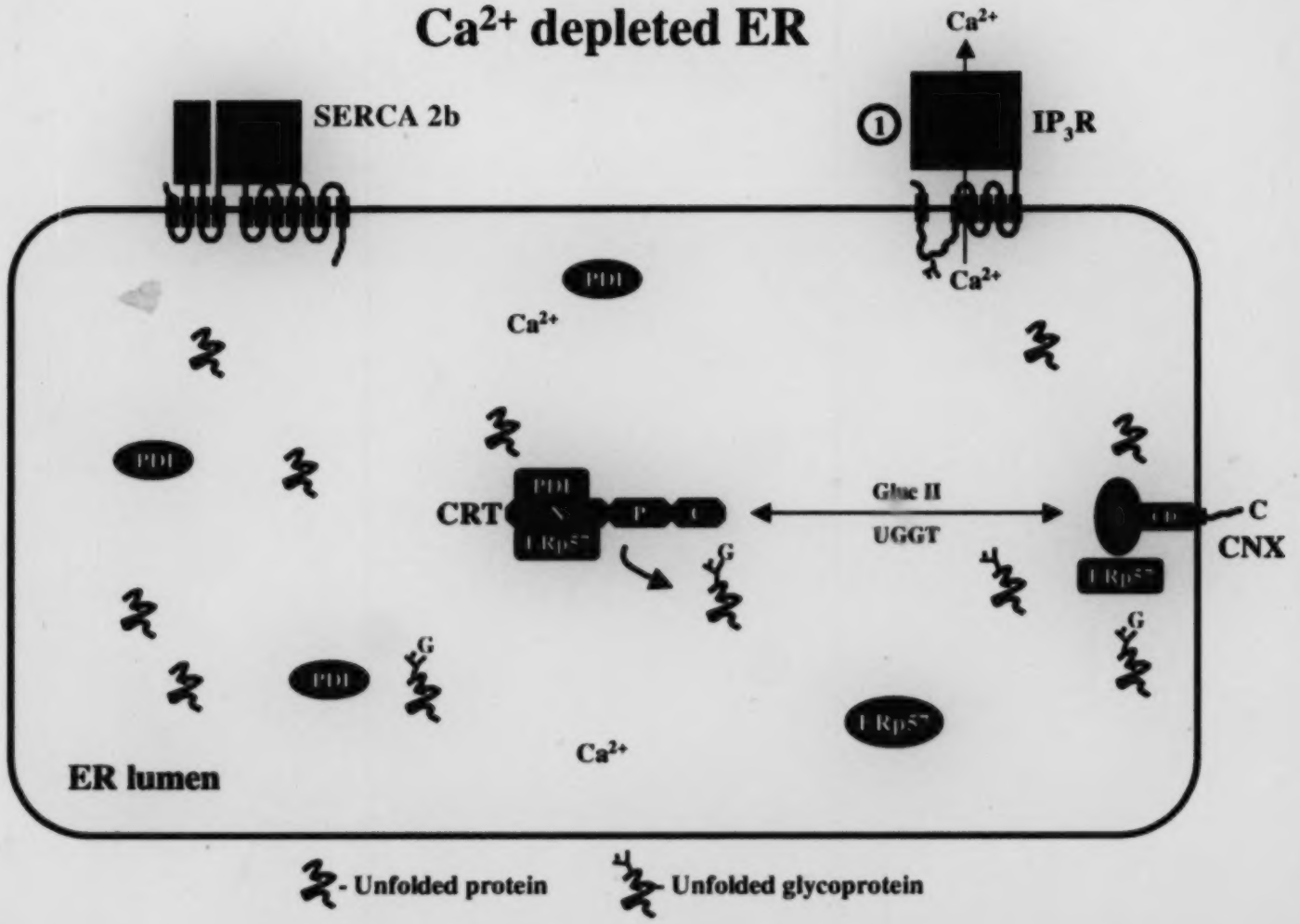
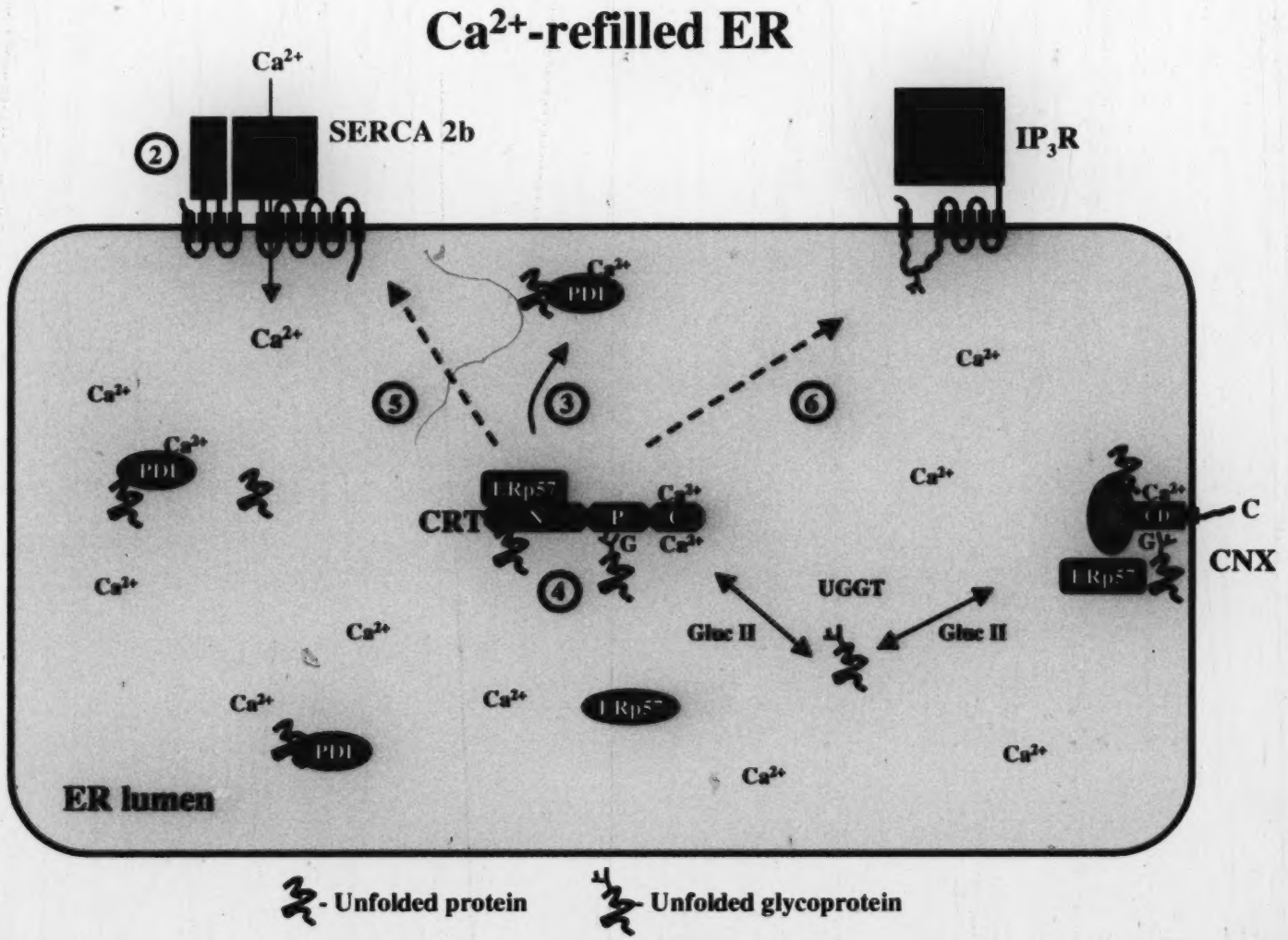

Ca2+ depleted ER
Ca2+
SERCA 2b
1
IP3R
Ca2+
Ca2+
PDI
Ca2+
PDI
CRT
PDI
N
P
C
ERp57
Gluc II
UGGT
C
CNX
ERp57
G
PDI
G
ERp57
Ca2+
ER lumen
- Unfolded protein
Unfolded glycoprotein

Figure 5-1. (B) Ca^{2+}-dependent dynamics in the lumen of the ER.

This diagram shows a model of events occurring in response to agonist activated Ca^{2+} fluctuations in the lumen of the ER. These events affect interactions between chaperones and the function of chaperones.

In B. (2) When Ca^{2+} is taken up by SERCA2b, ER stores are refilled ($[Ca^{2+}]_{ER}$ >400 μM) leading to full activation of chaperone function. (3) Interactions between calreticulin and ER chaperones (PDI) are regulated by Ca^{2+} binding to calreticulin. (4) Interactions between calreticulin and monoglucosylated unfolded glycoproteins are regulated by Ca^{2+} binding to calreticulin. (5) Calreticulin might also interact, in a Ca^{2+}-dependent manner, with the unique C-terminal tail of SERCA2b, to modulate its ability to refill ER Ca^{2+} stores. (6) Calreticulin might also interact with the $InsP_3$-receptor. Interactions between calreticulin (or calnexin) and misfolded proteins are also depicted. The role of Ca^{2+} in these interactions is not presently understood.

Broken arrows indicate that there is presently no direct evidence for interactions between calreticulin and SERCA2b and between calreticulin and $InsP_3$ receptor. Solid arrows indicate demonstrated pathways. The differing intensity of the background represents differences in Ca^{2+} concentration in depleted and refilled Ca^{2+} stores. CRT, calreticulin; CNX, calnexin; CD, central domain of calnexin; PDI, protein disulfide isomerase, Gluc II, glucosidase II; UDP-Gluc transferase, UDP-glucose:glycoprotein glucosyltransferase; G, glucose residue. Reprinted from *TiBS*, 25, Corbett, E.F., and Michalak, M. Calcium, a signaling molecule in the endoplasmic reticulum. 307-311.© 2000, with permission from *Elsevier Science*.


Ca2+-refilled ER
Ca2+
SERCA 2b
IP3R
2
PDI
ERp57
CRT
P
G
UGGT
Gluc II
Glu II
C
CNX
ER lumen
- Unfolded protein
Unfolded glycoprotein

interaction of chaperones with their substrates. The work presented here suggests that in agonist stimulated cells changes in the free $[Ca^{2+}]_{ER}$ affects inter-chaperonal interactions and may play a signaling role in the lumen of the ER and that Ca^{2+} binding chaperones could be involved in "sensing" these changes. It is widely accepted that Ca^{2+}, when released into the cytosol, is an extremely important signaling molecule. It is important to note however that this scenario may not occur in all cell types, but is applicable to agonist stimulated cells. Therefore in addition to its role in the cytosol Ca^{2+} should be considered an important signaling molecule in the ER lumen (Corbett and Michalak, 2000). With the generation of calreticulin-deficient fibroblasts it would be interesting to investigate the existence of such a system *in vivo*. Expression of different domains of calreticulin in calreticulin-deficient fibroblasts would allow us determine the role that the individual domains play in the chaperone function *in vivo*. It would allow us to examine interactions between calreticulin and other ER lumenal chaperones and substrates and the role played by Ca^{2+} in these interactions. Modulation of free $[Ca^{2+}]_{ER}$ would allow us to examine if such a similar system does in fact exist *in vivo*. Studies from our laboratory have already been initialized using these cells and given the first direct *in vivo* evidence that the C-domain of calreticulin plays an important role in Ca^{2+} storage and that the N-domain functions as a chaperone (Nakamura, 2001).

In order to gain more understanding of the dynamics of the ER lumenal environment and its influence on the functioning of calreticulin we investigated the role of Ca^{2+}, Zn^{2+} and ATP on conformational changes in calreticulin. We also investigated how interaction with PDI and ERp57 can influence calreticulins conformation in order to gain further insight into its biological role as a molecular chaperone.

As can be seen from the work presented in Chapter Three, binding of Ca^{2+} to calreticulin induced dramatic changes in the conformation of the protein. In order to examine more closely the structural changes occurring in calreticulin upon ion binding or chaperone interaction we examined the susceptibility of calreticulin to proteolysis in the presence of these factors (Chapter Four, Corbett *et al.*, 2000). When Ca^{2+} binds to the low affinity and high capacity C-domain the most C-terminal region of the protein appears to be extended and susceptible to proteolysis. The binding of Ca^{2+} to this region of the protein however results in dramatic conformational changes in the most N-terminal region of the protein and results in the formation of a 27-kDa protease-resistant core after proteolytic digestion, comprising all of the N-domain and some of the P-domain. Ca^{2+}-binding to the C-domain of calreticulin results in dramatic conformational changes in the N-domain of the protein, the domain that is involved in interactions with both PDI and ERp57 (Chapter Three). It is likely that these Ca^{2+}-induced conformational changes in calreticulin are responsible for the Ca^{2+}-dependent interaction of calreticulin with PDI and ERp57.

In addition to the Ca^{2+}-dependent interactions of calreticulin with PDI and ERp57, these Ca^{2+} induced conformational changes in calreticulin may have important physiological and pathophysiological consequences. This region of the protein is the most antigenic and digestion with elastase or cathepsin G – two of the major proteases released during inflammation - could result in cleavage of the C-terminus of the protein and the formation of this 27-kDa fragment *in vivo*. From the results presented here this fragment appears to be a highly stable structure (resistant to digestion over 6 hours) and might

explain why this is the most antigenic region of the protein and also provide a mechanism by which calreticulin is released into the circulation.

CD analysis (Chapter Three) and protease digestion (Chapter Four) of calreticulin clearly demonstrates that the binding of Zn^{2+} to calreticulin results in dramatic changes in protein conformation. Others have shown that Zn^{2+} binding increases the hydrophobicity of calreticulin (Saito *et al.*, 1999), and enhances its ability to suppress aggregation of non-glycosylated proteins. The conformational changes shown here, where the N- and C-domains of calreticulin are extended from the protein structure most likely expose hydrophobic regions of the protein that allow for this enhanced interaction with non-glycosylated folding proteins.

The data presented here demonstrates for the first time that calreticulin is indeed, like numerous chaperones an ATP-binding protein. ATP binding induces the most dramatic changes in conformation of the protein; conformational changes that expose hydrophobic regions of the protein and enhance calreticulins ability to suppress aggregation of non-glycosylated proteins (Saito *et al.*, 1999). Because Ca^{2+}, Zn^{2+} and ATP have all been shown to influence calreticulin-substrate interactions both *in vivo* and its conformation *in vitro*, conformational changes induced by these factors are relevant to the *in vivo* function of calreticulin as a molecular chaperone. It will be interesting to determine how Ca^{2+}, Zn^{2+} and ATP affect interaction of calreticulin with other ER lumenal chaperones *in vivo*. *In vivo* studies utilizing FRET may give a more definitive answer to this question. The work presented here suggests that ATP can bind to the C-domain of calreticulin. However, due to the lack of an ATP concensus sequence in

calreticulin it would be interesting to determine where exactly ATP is binding and if it represents a novel type of ATP-binding site.

That calreticulin be a flexible molecule in solution is consistent with its role as a molecular chaperone and its requirement to interact, however briefly with a large number of folding proteins in the ER. Results of this work clearly demonstrate that calreticulin is a flexible molecule in solution but also show that alterations in the dynamics of the ER environment influence its structure. These structural changes may explain how the ER lumenal environment can influence and perhaps control the function of calreticulin. The possibility that calreticulin is a flexible molecule in solution is also consistent with the protein being less amenable to crystallization. Previous experiments demonstrated that proteinase K or trypsin treatment of calnexin in the presence of Ca^{2+} yields a protease resistant fragment of the protein (Ou *et al.*, 1995). However all attempts to crystallize these fragments have proven unsuccessful thus far (Hahn *et al.*, 1998). The difficulty of growing crystals of the lumenal domain of calnexin was also attributed to the intrinsic flexibility of the protein (Hahn *et al.*, 1998). Attempts to crystallize calreticulin have also been unsuccessful. However the results presented here suggest that the addition of Ca^{2+}, Zn^{2+}, Mg^{2+}-ATP or a combination thereof may induce sufficient rigidity in calreticulin and allow the growth of crystals.

It is well established that Ca^{2+} is one of the most important signaling molecules in the cytosol – my work indicates that Ca^{2+} levels are also of central importance in dictating the function of proteins in the ER. Thus Ca^{2+} may also have been chosen as a signaling molecule in the ER. The work presented here indicates that calreticulin "senses" changes in the free $[Ca^{2+}]_{ER}$, *via* its high capacity Ca^{2+} -binding site, and, in

response to these changes, alters its interactions with other chaperones and Ca^{2+} binding to its high-affinity Ca^{2+}-binding site affects its interaction with unfolded glycoproteins. Calreticulin, therefore, could be a key player in Ca^{2+}-signaling processes within the ER.

Protein interactions are essential to virtually every cellular process and pathway. Protein interactions can affects cellular processes in different ways - inducing conformational changes in a protein (Chapter Three), cause changes to the kinetic properties of a protein or possibly create a new binding site for substrates. Protein-protein interactions can either stimulate or inhibit the function of a protein (for example, the enhancement of ERp57 disulfide isomerase activity in the presence of calreticulin). Understanding protein-protein interactions occurring between specific molecules allows for the understanding of the mechanisms involved in a given cellular process. Therefore the study of calreticulin chaperone-chaperone interactions *in vitro* will provide insight in to the functional role that it plays in a cell and how its activity is regulated.

Calreticulin is widely accepted to be a multifunctional protein with numerous diverse cellular functions. The identification of proteins interacting with calreticulin and the factors that can influence these interactions will help clear some confusion as to how exactly one protein can perform so many functions! As our understanding of how calreticulin performs all of its different roles becomes more complete we can examine the possibility of using this information to our advantage.

Chapter Six

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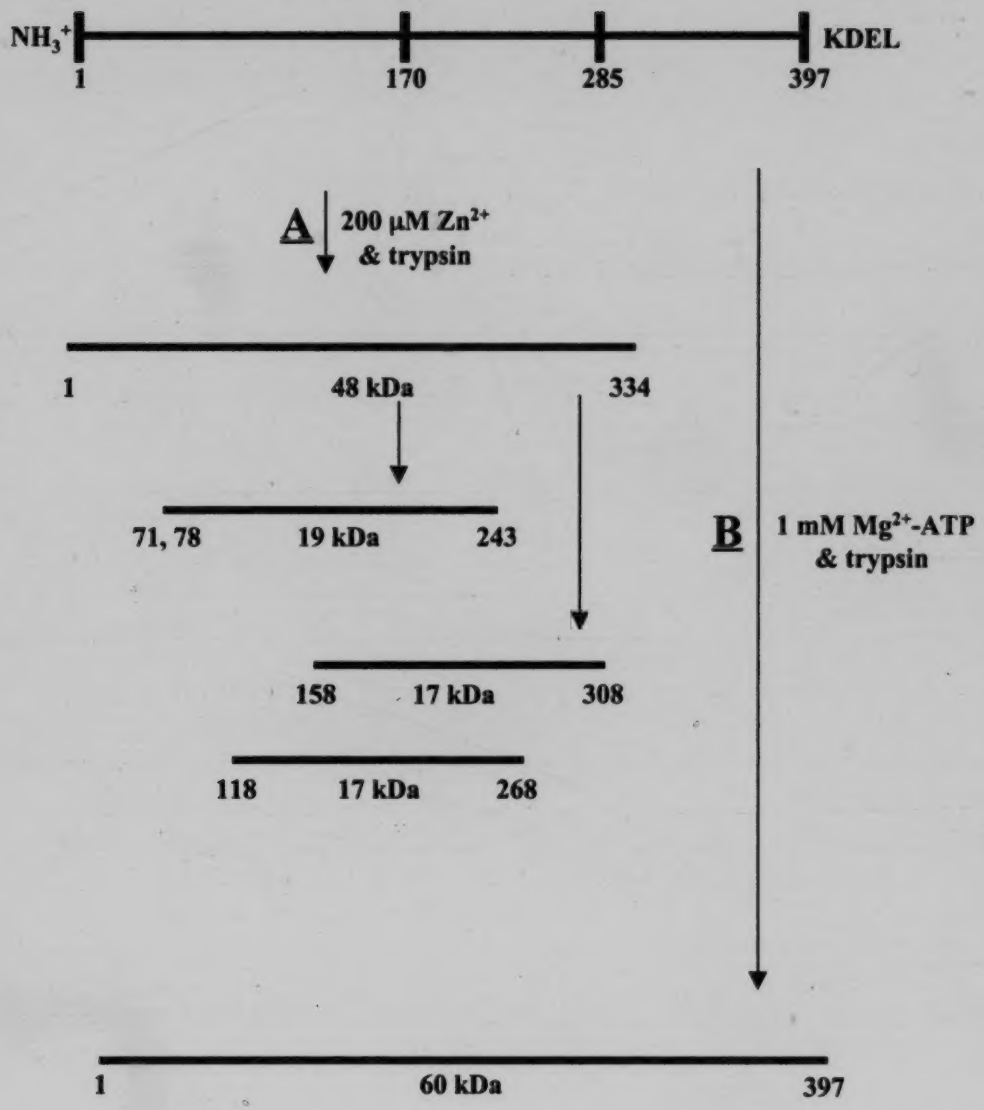
Appendices

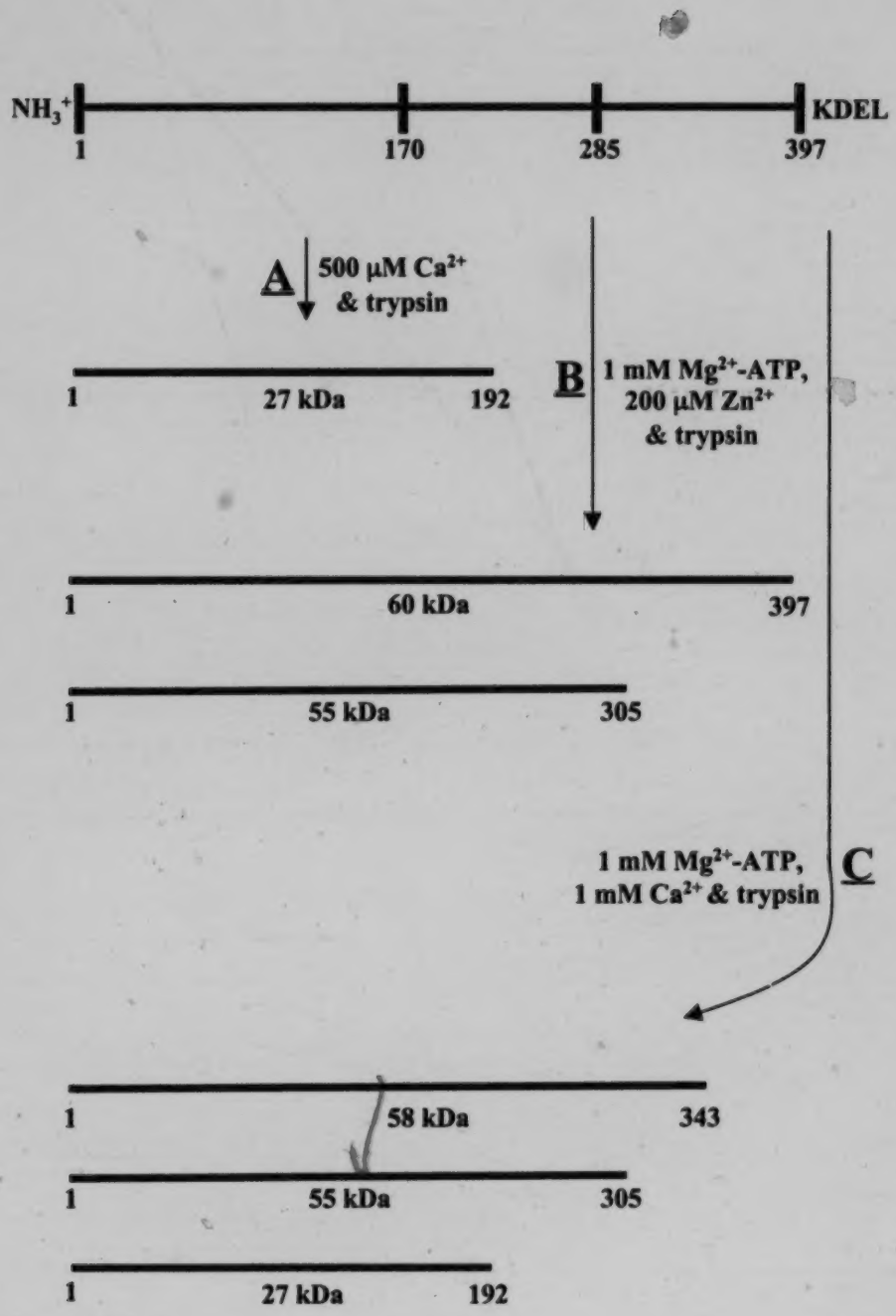
Appendix 1. Model for tryptic digest of calreticulin in the presence of Zn^{2+} & Mg^{2+}-ATP.

The following is a schematic representing the protease resistant fragments of purified calreticulin after digestion with trypsin (Chapter 4). Digestion of calreticulin in the presence of: (**A**), 200 μM Zn^{2+} (**B**), 1 mM Mg^{2+}-ATP. The amino acid identity at the N-terminus of the fragments was determined by N-terminal amino acid sequencing, while the identity of the most C-terminal amino acid is an approximate.


NH3+
KDEL
1
170
285
397
A
200 μM Zn2+
& trypsin
1
48 kDa
334
71, 78
19 kDa
243
158
17 kDa
308
118
17 kDa
268
B
1 mM Mg2+-ATP
& trypsin
1
60 kDa
397

Appendix 2. Model for tryptic digest of calreticulin in the presence of Ca^{2+}, Mg^{2+}-ATP & Zn^{2+} and Mg^{2+}-ATP & Zn^{2+}.

The following is a schematic representing the protease resistant fragments of purified calreticulin after digestion with trypsin (Chapter 4). Digestion of calreticulin in the presence of: (A), 500 μM Ca^{2+}, (B), 1 mM Mg^{2+}-ATP & 200 μM Zn^{2+}, (C), 1 mM Mg^{2+}-ATP & 1 mM Ca^{2+}. The amino acid identity at the N-terminus of the fragments was determined by N-terminal amino acid sequencing, while the identity of the most C-terminal amino acid is an approximate (an exception is the 27-kDa protease resistant fragment in (A) and (C), where the C-terminal amino acid is known.


NH3+
KDEL
1
170
285
397
A
500 μM Ca2+
& trypsin
B
1 mM Mg2+-ATP,
200 μM Zn2+
& trypsin
1
27 kDa
192
1
60 kDa
397
1
55 kDa
305
C
1 mM Mg2+-ATP,
1 mM Ca2+ & trypsin
1
58 kDa
343
1
55 kDa
305
1
27 kDa
192